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Laboratory Diagnosis *and* Experimental Methods *in* Tuberculosis

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LABORATORY DIAGNOSIS
AND
EXPERIMENTAL METHODS
IN
TUBERCULOSIS

Laboratory Diagnosis *and* Experimental Methods *in* Tuberculosis

By

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HENRY STUART WILLIS

The Johns Hopkins University and Hospital

WITH A CHAPTER ON

TUBERCULO-COMPLEMENT FIXATION

By

J. STANLEY WOOLLEY

Loomis Sanatorium

LOOMIS, NEW YORK

INTRODUCTION

By

ALLEN K. KRAUSE

The Johns Hopkins University

CHARLES C. THOMAS
SPRINGFIELD, ILLINOIS - BALTIMORE, MARYLAND
1928

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Dedicated *to two teachers whom I hold*
in sincere appreciation and
affectionate regard:

Dr. Allen K. Krause
of the Johns Hopkins University

and

Dr. H. V. Wilson
of the University of North Carolina

each of whom has, by word and deed and
published work, been a source
of encouragement and
stimulation

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PREFACE

This book has a twofold purpose. It aims to describe the more important methods of the laboratory diagnosis of tuberculosis and to consider some of the more significant procedures and principles involved in the experimental study of the infection. In the first instance, it has brought together in the compass of one chapter a consideration of the fluids, secretions and excretions of the tuberculous body. Following this, it enters into an exposition of methods for the detection of the tubercle bacillus by staining, isolation and animal inoculation, gives a description of this microörganism and compares it with other acid-fast bacteria and deals with serological diagnosis and diagnosis by the use of tuberculin. Secondly it devotes several chapters to some of the general factors concerned in the infection of animals and the study of tuberculosis experimentally.

The guiding principle has been to cover the subject as concisely as is consistent with adequacy of presentation. Therefore, I have sought to present clearly in each of the various parts of the book only so many of the available technical procedures as appeared commensurate with the immediate subject at hand, always with an eye to the conservation of space and prevention of too much duplication of essentially similar methods. Of the methods outlined, most have been proved dependable through long application, but a few have been included which are new and virtually untried, because selection has occasionally been made on promise and rationale as well as on demonstrated worth. This plan has led to the omission of many methods, some of them of unquestioned value.

It has been my desire to write a book which would be useful to practising physicians, to public health officials and laboratory workers in general and to medical students. And in its preparation I have had recourse to a number of friends for criticism, suggestion and review: To Dr. J. Burns Amberson, Jr.

for criticism from the viewpoint of the sanatorium physician; Dr. Allen K. Krause who originally suggested the book and who, in addition to writing the introduction, has made many valuable suggestions as to plan; Dr. William A. McIntosh for review from the standpoint of the public health officer; Dr. William Snow Miller for permission to describe some of his valuable technical procedures in the care and staining of tissues; Dr. Arnold R. Rich for suggestions that might occur to a pathologist; Dr. J. Stanley Woolley, who has had an extensive experience with serological diagnosis of tuberculosis, for helpful ideas from the point of view of the sanatorium-laboratory, and also for the writing of Chapter X on complement fixation; to Drs. A. N. Aitken, Charles R. Austrian, Arthur L. Bloomfield, W. H. Lewis and M. R. Lewis, W. A. Perlzweig and Max Pinner for helpful suggestions on various questions throughout the work; to these I make grateful acknowledgment. I have also to acknowledge the faithful and cheerful coöperation of Misses Mary E. Bachman, Thelma Leach and Abra Ella Spicer, who have copied and recopied parts of the manuscript many times. The last of these has also helped in the reading of proof.

H. S. W.

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INTRODUCTION

By Allen K. Krause

INTRODUCTION

At the very outset of his quest for the tubercle bacillus Koch learned that the tubercle bacillus was a rather special kind of germ. With simple implements,—cover-slip smears, methylene-blue dye, gelatine tubes and plates,—he approached the new Tree of Knowledge; to such effect, indeed, that, as he put it himself, in those first flush years bacteria fell into his lap "like ripe apples from a tree." It had been in 1876 that he carried up the life-story of *Bacillus anthracis* to Ferdinand Cohn at Breslau. It was in December, 1881, when he essayed the snaring of the elusive virus of tuberculosis. He first applied to the task his now well-tested and fruitful methods. For the first time they failed him. But, rising to the occasion, his genius now directed him to other men's devices, used in breaking other paths, which by March, 1882, permitted him to observe the entire cycle of development of the tubercle bacillus, a germ which, in staining, in appearance, and in ways of growth was different from all bacteria then known.

The years since have served to enhance for the tubercle bacillus its early reputation for singularity. Protean, versatile, universal and catholic it might be in effects, whether pathological or clinical; so much so, indeed, that there are still many who find it difficult to comprehend all that passes for *Tuberculosis* as due to one and the same microörganism; but zoölogically (or botanically ?) it appears to stand in a class by itself, possessing well-defined biological peculiarities, or, at the most, constituting a type to which a company of described forms may be correlated.

This circumstance, in itself, would naturally promote specialization in dealing with the tubercle bacillus bacteriologically, a tendency that was decisively directed by the gradual though unceasing trend toward specialization of the clinical contact with tuberculosis. Not a few of us may have deplored the setting apart of tuberculosis as a field for practice;

it is none the less true that neither the certainty nor the momentum of the movement has been abated a jot by all our criticisms and lamentings. Tuberculosis as a medical specialty is here to stay,—at least as long as there remains enough manifest tuberculosis to be diagnosed and treated with personal profit. Tuberculosis as a specialty of the laboratory is perhaps on its way.

Let us hope that the day of its arrival will never come; for the new era could hardly contribute to the healthiness of either tuberculosis or general medicine as a subject to be cherished and developed progressively and scientifically. We like to believe that, the greater tuberculosis grows in scope, the more surely it must merge with general medicine,—the more certainly each must be elucidated through familiarity with the other.

Yet, it is not to be doubted that we have now passed the time when the needs of the worker, interested with any of the myriad facets of tuberculosis, can be best served by a multitude of unrelated sketches and references scattered over and throughout a terrain that is as long and broad as the multipartite divisions of modern medicine. A germ that habitually bears down upon almost every organ and tissue of the body necessarily creates a corpus of diagnostic method that is commensurate with the unrivaled multiplicity of its pathological and clinical manifestations. The works on urology will present their share of the diagnostic procedures of tuberculosis, the works on orthopaedics theirs, on gastroenterology theirs, and so on; until to-day the laboratory diagnosis of the infection can be got at only in discouragingly piecemeal fashion by practitioner, student, or professional technician. For bringing together within a single volume these numerous diversified methods, and for appraising them to the enlightenment and profit of the unspecialized worker, Doctor Willis deserves the thanks of the medical profession.

Doctor Willis has also performed a long-needed service in presenting in orderly manner the principles and procedures of experimental method in tuberculosis. He has developed this section in so simple yet adequate a manner that the cause of tuberculosis research should be greatly furthered by those young workers on the threshold who may presumably be encouraged to enter the inner domain of productive study.

The diagnosis of tuberculosis remains so wofully imperfect to-day not because of paucity or inadequacy of method. Method we have in abundance,—good method,—method sufficient for an extraordinarily high average of proficiency. We have clinical method enough and laboratory method enough for first-class attainments. Where we are lacking is in performance. What is wrong lies almost wholly in the great numbers of practitioners who are too busy or uninterested to secure satisfactory clinical histories; too indolent to use the stethoscope or too unpractised to employ it understandingly; too neglectful to keep at the microscopic examination of specimens, or the tuberculin testing of the skin; too ready to throw the entire burden of diagnosis on the X-ray. Experience continues to teach that the thoroughly useful methods in the diagnosis of tuberculosis are not the difficult, the complicated, the cumbersome, the recondite ones,—the ones employable only by the "specialist." It is safe to say that a fourth-year medical student can diagnose 80 per cent, and better, of all *diagnosable* cases of pulmonary tuberculosis through the employment of a few, a very few, simple and well-tried manoeuvres,— *if only he will use them*. It is to be hoped that this book by Doctor Willis will drive home this central theme; and that one important result will be to rid a respectable proportion of practitioners of an "inferiority complex" which they seem to have developed when confronted with the necessity of a diagnosis in which tuberculosis may figure. The book will have been worth while if it succeeds in teaching that the laboratory diagnosis of the ordinary case of tuberculosis can be accomplished by simple methods in an ordinary physician's hands in an ordinary physician's office.

A. K. K.

PART I

GENERAL CONSIDERATIONS OF THE BODY FLUIDS AND EXCRETA

CHAPTER I

DESCRIPTION OF THE SEVERAL SECRETIONS, EXCRETIONS AND CONSTITUENTS OF THE BODY IN TUBERCULOSIS

The tubercle bacillus may attack and alter any tissue in any part of the body. It is obvious, therefore, that a familiarity with the general properties of all of the available secretions, excretions and fluids of the tuberculous body is essential for a thorough laboratory study of the disease.

While it is the fact that few, if any, of these fluids, secretions and excretions of the body have properties which are specific for or pathognomonic of tuberculosis, most if not all of them do possess certain features which approach specificity and certain properties which help to point the way to the diagnosis. It is these characteristics and the methods at our disposal for detecting them which will be dealt with in the major part of this book. It has been found convenient to present these considerations in two divisions. Part I enters into a general description of the fluids and the means of obtaining them, while Part II gives a detailed account of the technique of demonstrating the tubercle bacillus in these materials by staining and concentration methods and by animal inoculation.

It may be proper at the outset to call attention to the importance of having the glassware used for collecting specimens scrupulously clean. Only containers should be used which are new or which have been treated with a cleaning solution such as the following one:

Potassium bichromate............... 75 grams.
Water............................200 cc.

Heat until dissolved and then allow to cool
Add *very* slowly,
Concentrated sulphuric acid..........400 cc.
Stir gently but frequently.

The amount of sulphuric acid is relative. It should be added until crystals form.

Tubercle bacilli are killed by boiling or autoclaving but their morphological identity is not destroyed by such means. Dead but intact bacilli may therefore adhere to containers or slides, be stained and in this way become a source of serious error. This certainly does not occur commonly, but it is serious and should never occur.

SPUTUM

1. *General characteristics:* Sputum in pulmonary tuberculosis is not typical of the disease, as this secretion often is in bronchiectasis, asthma or abscess of the lungs, yet it does possess certain features to which attention should be called. Sputum from a patient with pulmonary tuberculosis is often, perhaps most often, grayish-yellow, glairy, viscid, tenacious, mucopurulent or purulent. The mucopurulent lumps are often suspended in a thinner, more liquid material, but more commonly they settle to the bottom of the container and sometimes assume a rather globular shape. These gobs of sputum are not always homogeneous, for not uncommonly they contain numerous minute, white or grayish-white particles ranging in size from a speck at the point of vision to a diameter of about one millimeter. Less sticky than the matrix in which they lie, they give the impression of granular paste when smeared on the slide. They are important because they are composed either of small aggregations of pus cells or fragments of necrotic pulmonary tissue. They are much more likely to contain tubercle bacilli than the more mucoid elements of the specimen are, and, when present, should be selected for smear and stain. When these specks cannot be seen and selected, as occasionally happens, their detection may be facilitated by squeezing a portion of the specimen between two large glass plates against a black background. Such a procedure also often reveals the presence of elastic tissue—a sure sign of destruction of the lung. The method is messy, however, and, unless precautions are observed, is more likely to spread bacilli around than methods which confine the sputum to the container.

Since tubercle bacilli are nearly always demonstrable in the pus which comes from tuberculosis of the lung, the persistent presence of pus in sputum in which careful and repeated search fails to reveal these germs is presumptive evidence against tuberculosis and points rather to abscess of the lung, bronchiectasis, a mycotic infection or some other nontuberculous disease. It is obviously important to detect the causative bacteria in such sputum (see page 121).

In the course of pulmonary tuberculosis, the patient may occasionally cough up small pneumoliths. These stones, white and chalky and of irregular outline, represent deposits of calcium in areas that were once composed of necrotic tissue. They may be very minute or they may be as large as a centimeter in diameter. Large numbers may be coughed up especially during periods of haemorrhage.

2. *Quantity:* In the early stages of pulmonary tuberculosis or in the very chronic, fibroid types of the disease, there is, as a rule, but little sputum. Particularly in the latter instance there is likely to be a little in the early morning only. Usually the production of the sputum is associated with a slight cough, although at times the cough may be severe and exhausting and the sputum scant. If the sputum is scanty in amount, the total 24-hour specimen should be saved. The amount may be increased by the administration of five or ten grains of potassium iodide, three times a day, for three or four days. Following such treatment, bacilli occasionally appear in the sputum when they have not been demonstrable before. Bloody sputum may appear (rare) after administration of this drug.

The amount of sputum varies greatly in different individuals and it may also fluctuate somewhat from time to time in the same person. When the total 24-hour output is measured or weighed daily or weekly over a considerable period, its variation has some significance. A rapid increase of total sputum often occurs with breaking down and excavation of tissue, and a continuous, prolonged increase should cause one to suspect progressive cavity formation. When there is a cavity, a gradual decrease in the amount of sputum may be taken as a probable index of healing and shrinking of the lesion. Such observations assume considerable importance if used as corroboratory evidence along with changes in symptoms, signs and roentgenograms of the patient. Sudden changes may arise also from other causes (Brown (27)). For instance, with the onset of an intercurrent infection, cardiac disease and sometimes miliary tuberculosis, there is a decrease in the amount of sputum although at times the miliary form of the disease is initiated by a considerable increase in sputum-output. Sputum may completely disappear for a week or longer after the onset of tuberculous pneumonia; toward the end of this time it gradually increases to a point ordinarily far in excess of that prior to the pneumonia. Bacilli may likewise disappear for a few days, even when all sputum-elimination is not suppressed; only to reappear later.

3. *Chemical change:* Much importance has been attached to the albumin content of the sputum in tuberculosis. Formerly it was thought that the finding of a little albumin in sputum always indicated tuberculosis of the lungs. But it is now known that albumin *may* be present in the sputum in any inflammatory disease of the respiratory tract including the pleura. Moreover, it is sometimes absent in the sputum of the tuberculous. Its presence is considered by some to be accessory evidence of tuberculosis, but it is of little practical value. The test is easily made by first eliminating the mucus by acetic acid or by fermentation (Pottenger (181)) and then examining for albumin by the ordinary method of heat and acetic acid as in testing for albumin in the urine.

4. *Haemorrhage:* Looked upon by many of the older physicians as a cause of phthisis, pulmonary haemorrhage was thought to prepare the soil for the disease by the retention and clotting of the blood within the lungs. The explanation of this ancient interpretation rests on the well-known clinical observation that haemoptysis frequently ushers in tuberculosis in seemingly healthy people. It is a common observation clinically that, a few days after an haemoptysis, the patient may (1) show his first evidence of illness by developing signs and several of the symptoms of pneumonia; or (2) he may show an exacerbation of previously existing symptoms and an increase

in signs of his preëxisting tuberculosis; or (3) he may experience no change in symptoms and his chest reflect no alteration in signs before and after haemorrhage. Recent work by Austrian and Willis (8) would tend to show that in the first instance large numbers of tubercle bacilli from the focus are spilled with the blood into the neighboring pulmonary tissue and that the body, being allergic by virtue of being infected (see p. 260), responds with the formation of an exudative pneumonia; that in the second instance fewer bacilli are distributed with the blood and a lesser degree of inflammation occurs; and that in the last named circumstance no bacilli are spilled with the blood.

Haemoptysis occurs in probably more than one-half of the people with pulmonary tuberculosis, and the amount of blood may vary from a very few small, just perceptible specks or streaks in the sputum to a huge volume which may eventuate in the death of the patient either by exsanguination or suffocation from plugging of the trachea. It may appear at any stage of pulmonary tuberculosis, not infrequently ushering in the disease, but is much more common in advanced cases.

While ordinarily it is easy to determine that the blood has come from the lungs, there are times when its source is debatable and the demonstration of the source important. As a rule, blood from the lungs is coughed up; it is bright red and usually somewhat foamy; it is alkaline in reaction when fresh, and frequently the centrifugalized specimen contains tubercle bacilli, fragments of elastic tissue or débris from a cavity. For a day or two soft clots are brought up which tend to become dry and of a dark color after the first few hours. On the other hand, blood from the stomach is vomited. It generally has a dark color and is lumpy or clotty; unless in large quantity and very fresh, it has an acid reaction and is likely to contain particles of food. Blood that comes originally from the lungs may be swallowed and later vomited, in which case both the past history of the patient and the story of the actual occurrence of the haemorrhage itself help in making the diagnosis. Such blood may likewise contain tubercle bacilli. Auscultation will occasionally reveal râles at the site of the haemorrhage. In the sputum from chronic pulmonary bleeding, "heart-failure" cells are not uncommon.

Although haemorrhage may occur in several diseases other than tuberculosis, that from cardiac failure is the chief one in which there may be serious confusion with the blood from pulmonary tuberculosis. But in such circumstances, there are never any tubercle bacilli or elastic tissue in the blood or sputum, while "heart-failure" cells may be easily demonstrated. And clinically the history, symptoms and signs especially the bilateral, basal location of râles even in the absence of abnormal signs in the heart itself, ought to clear up the difficulty fairly readily. In the various other pulmonary diseases with haemorrhage, the physical and roentgenological examinations usually point the way, except in certain of the mycoses; and here careful, painstaking microscopic and cultural study of the sputum will yield up the secret in most instances.

5. *Method of obtaining:* The method of obtaining specimens of sputum is important, particularly in early cases, in which it is often desirable for the doctor to superintend the collection so as to be reasonably sure that the specimen is coughed up from the lungs and not "raised" from the nasopharynx. The specimen should be obtained in the early morning whenever possible because, at any stage of the disease, the morning sputum contains a larger number of tubercle bacilli, or is more likely to contain them, than that obtained at other times during the day. If sputum is to be examined soon after its collection, it may be deposited in any of the numerous types of paper, cardboard or wooden box that are in common use in sanatoria, hospitals and dispensaries. But if the sputum must remain in the container for a day or two or if it must be sent through the mail, it should be collected in a wide-mouthed bottle of clear glass made without a neck and fitted with an unused cork. Bottles with a narrow neck or those made of colored glass should be avoided, for the obvious reason that it is difficult for the examiner to see the specimen and to "get at" it. It is sometimes more satisfactory to make smears of the specimen after it has been poured from the container into a petrie dish which sits on a black background. No matter what

the type of container, it should never be used more than once.

It is to be borne in mind that in infants and children it is often difficult or impossible to obtain a specimen except by swabbing the throat or nose after cough, or by examining the gastric secretion or the stool.

URINE

1. *General remarks:* In extraurogenital tuberculosis the urine reveals a few variations from the normal, but they are of no diagnostic import. Diuresis when the patient is put at rest on a large intake of milk; oliguria late in the disease with diminishing fluid intake and profuse sweating; a little albumin and occasionally a few granular and hyaline casts in fever, during absorption of pleural effusions, or in rapidly progressing or in very chronic pulmonary tuberculosis when amyloid disease supervenes; or occasionally when a true nephritis appears quite independently of tuberculous disease; glycosuria if diabetes and tuberculosis are associated, as not infrequently happens: One or several of these features are fairly often encountered. Renewed emphasis has been recently put upon the diazo reaction which, while not specific, is nearly always present in the very late stages of the disease (Lemmens (123)). When present, it indicates a fatal issue within a few months.

2. *Characteristics:* In the diagnosis of urogenital tuberculosis, several factors aside from the actual examination of the urine are of significance. For instance, the diagnosis is advanced by the family or personal history of tuberculosis or the presence of any one of several conspicuous symptoms such as frequency (the commonest), haematuria, pyuria, dysuria or urgency (239). Unexplained polyuria or haematuria which is slight, intermittent or recurrent, especially if it is associated with slight pyuria, bladder symptoms or vague lumbar pain, should cause one to examine the urine carefully for tubercle bacilli. The urine may be normal in gross appearance; it may be slightly turbid from a beginning pyuria; or it may have the smoky appearance which is associated with mild grades of haematuria. Pus-containing urine in which microscopic examination fails to reveal any microörganisms of disease is a leading

sign of tuberculosis, because in nearly every other instance in which pyuria is a symptom of urogenital infection, the germ responsible for the infection is easily demonstrated. But it does not follow from this that the detection of non-acid-fast bacteria in purulent urine necessarily rules out tuberculosis, because secondary invaders, particularly the colon bacillus, occasionally inhabit the urine in cases of urogenital tuberculosis. Moreover, it should be borne in mind that pyuria without demonstrable pathogenic microörganisms sometimes occurs in posterior urethritis. Acid-fast bacilli are sometimes scanty and sometimes present in enormous numbers in the urine—large numbers speaking strongly for the human and against the smegma bacillus. Just as with pyuria and haematuria, bacilluria may also be intermittent, so that examination of repeated specimens may be necessary before they are found. Demonstration of bacilli in the urine is naturally of the greatest moment, and it is important in the quest for them to employ methods that yield reliable results. A thorough discussion of genito-urinary tuberculosis is given by Young and Davis (239).

Bacilluria has been reported in miliary tuberculosis, in tuberculous meningitis, and in a few patients with pulmonary but without urogenital tuberculosis. Hobbs (86) in a recent study of 100 cases of pulmonary tuberculosis without clinical signs of renal disease, showed, by animal-inoculation of single catheterized specimens of urine, that bacilli were present in six. But he also found microscopic tuberculosis in the kidney in 6 of 18 of these 100 cases which subsequently came to autopsy. It is, therefore, difficult from these figures to be certain whether the bacilli in the urine came from kidneys which were free from tuberculous disease. The absence of renal disease in such conditions would be difficult to prove without inoculation of a large bulk of renal tissue. Such a circumstance, if it does occur, must be regarded as rare, and tubercle bacilli in the properly collected urine means, for all practical purposes, renal or urogenital tuberculosis.

3. *Method of obtaining: preparation for study:* Smegma bacilli normally inhabit the genitalia and skin of man. Indistinguishable morphologically and tinctorially from tubercle

bacilli, they may occasionally become a troublesome factor in the diagnosis of genitourinary tuberculosis. For this reason, dependence upon ordinary smear preparation is notoriously unreliable, at least theoretically so. Fortunately, however, a scheme which eliminates confusion or uncertainty in this respect has been worked out by Churchman (37). It consists of the following steps. After the external genitalia have been thoroughly washed and the external urethra irrigated several times with sterile water, urine is collected in three separate sterile vessels, the first and third of which are discarded and the second saved for centrifugalization and microscopic examination. It may, however, be collected in two vessels and the

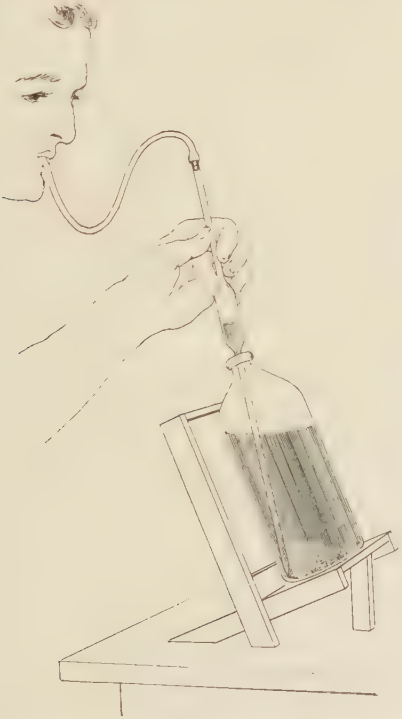

Figure 1. Drawing of Support for Allowing Sedimentation of Urine
The incline causes all the sediment to collect in the lowermost portion of the bottle where it is easily obtainable by pipette.

second one saved for examination. Any acid-fast bacilli that are present in such specimens are tubercle bacilli, and bilateral catheterization will show from which kidney they come. In gynecological practice, careful cleansing of the external genitalia is followed by catheterization of about 50 cc. of urine for examination. Bacilli, however, are often less numerous in the bladder urine than in that obtained directly from the pelvis of the kidney. Bacilli in urine collected in this way are always tubercle bacilli and further catheterization will usually indicate their source.

These methods of obtaining the specimen require a good deal of special skill, which it is possible to forego by collecting the urine for twenty-four hours under careful conditions. A sterile 2-liter acid bottle is subjected to the action of cleaning solution (p. 31), 10 or 15 cc. of toluol[1] is put into the bottle as a preservative and the bottle kept in the ice chest, in a plane about 30° from the vertical on a convenient frame such as shown in figure 1. Specimens are collected in a urinal known to be free from tubercle bacilli and added to the bottle through a funnel of similar cleanness. If the patient have bacilli in his sputum, care should be exercised lest he contaminate the specimen or container. After the addition of the last specimen, the bottle is kept in the ice box for several hours. By virtue of the inclined position there is a slow, gradual settling of all particulate matter, precipitated salts, bacteria, etc., toward the lowermost "corner" of the bottle. Twenty-five to 50 cc. of the sediment is withdrawn by means of a long volumetric pipette for digestion or centrifugalization and examination (p. 54). Acid-fast bacilli found in the specimen may be either pathogenic or parasitic. If in quantity, they are almost certainly pathogenic. Animal inoculation should be done. But meantime bilateral catheterization and search for bacilli will in most instances settle the diagnosis.

Instead of this method, a little 30 per cent acetic acid may be added to the 24-hour specimen, followed by the addition of 2 cc. of 5 per cent tannic acid for each liter and the bottle allowed to stand a few hours in the ice chest, whereupon the sediment is removed and digested with 4 per cent sodium hydroxide (Petroff (14)) and then examined.

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[1] Toluol is superior to chloroform as a preservative.

It is desirable to use a 24-hour specimen for two reasons. In the first place tubercle bacilli may be eliminated intermittently with the consequence that a single specimen may contain but a few or none. In the second place, the sediment from so large a quantity of urine should contain tubercle bacilli in greater numbers than that from a single specimen, which fact would facilitate their detection.

When there is tuberculosis of the genital system, prostatic massage frequently yields secretions that contain the offending bacterium. Before massage, however, the glans and meatus should first be cleansed and the urethra irrigated. When this precaution has been taken, any acid-fast bacilli found may be depended upon to be tubercle bacilli. If there is a suspected tuberculous process in the epididymis and no bacilli in the prostatic secretion, aspiration through the scrotal sac usually yields sufficient matter for smear and stain (Young).

STOOL

1. *General remarks:* Tuberculous enteritis, once thought of only as a not uncommon terminal event in the course of pulmonary tuberculosis, has been elevated to the status of a clinical entity with a prognosis that is not necessarily always grave. This has come about through the work of Brown and Sampson (28) and Paterson (172), among several others. Many mild cases which earlier went without recognition and probably underwent spontaneous healing are being detected. We are now told that there may be no diarrhea in the disease or that it may come only late in the progress of the malady. The patient may have only one stool a day, but, instead of being of the normal consistence for the patient, it is a little soft. Or there may be a soft stool twice a day. In different patients there will be seen every gradation in the stool, both as to frequency and consistence, from a normally formed stool once a day, through the soft or "mushy" stool once or several times a day, to the most extreme watery diarrhea. Remissions and alterations in the type of stool are frequently observed. Since

the lesions are commonly ulcerative in character, flecks of mucus and pus and, less commonly, macroscopic blood may be intermixed with the soft or semi-formed stool. But tuberculous enteritis frequently exists without the appearance of these substances.

The examination of the stool itself is not very helpful, except in a negative way, and the clinician must depend almost entirely upon symptoms and roentgenograms for the diagnosis. However, the failure to find tubercle bacilli in the stool after repeated examination points away from the diagnosis of intestinal tuberculosis. On the other hand, the discovery of these germs in the stool by no means necessarily indicates the presence of this disease, because at some time during the course of open pulmonary tuberculosis, probably every one who has the disease swallows tubercle bacilli. This statement rests on the results of several careful studies such as that of Bodo (19) who in 1891 found acid-fast bacilli in the stool from three of nine patients having similar bacteria in the sputum and who revealed no sign of intestinal tuberculosis at autopsy; that of Klose (92) in 1910 who found tubercle bacilli in the stools from 55 out of 60 patients having a positive sputum; that of Laird, Kite and Stewart[2] (119) who concluded that nearly all persons with a positive sputum had also a positive stool. In most instances these bacilli undoubtedly pass along the entire length of the digestive tract and are eliminated without ever initiating lesion, while only occasionally conditions may be favorable for them to set up enteric tuberculosis.

However, there are times when the discovery of bacilli in the stool is of the greatest value. Such occasions are encountered in children suspected of having pulmonary tuberculosis from whom a specimen of sputum cannot be obtained, or in adults who either wittingly or unwittingly swallow the pulmonary secretions.

The stool is usually differentiated from that of the dysenteries, diarrheas, etc., by the history, the clinical character of the malady, the presence of an aetiological agent or the presence of pulmonary tuberculosis.

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[2] The latter authors have given a good review of the literature.

2. *Method of obtaining: preparation for study:* In the earlier days of the bacteriological era, tubercle bacilli were sought in the watery stools of advanced intestinal tuberculosis, and it was recommended by Rosenblatt (188), in 1899, that the patient be given laudanum until the stools became sufficiently hard to scrape off or detach flecks of mucus and pus in rubbing against the ulcerated areas. The hard stool was then searched for these flecks or gently rubbed and the material obtained transferred to slides and stained for tubercle bacilli. Such procedures have long since been superseded by better ones, such as that of Petroff (176), who has devised a very satisfactory means of detecting tubercle bacilli from the stool by subjecting it to the action of sodium chloride followed by sodium hydroxide. The stool contains numerous spores which resist the action of sodium hydroxide and this causes the length of time of incubation, therefore, to be a matter of importance. The following are Petroff's directions:

> Collect fresh specimen of stool in a clean, wide-mouthed jar.
> Add three volumes of water and thoroughly mix.
> Filter through several layers of gauze.
> To the filtrate add sodium chloride to saturation.
> Let stand 1/2 hour, when all bacteria will be floating.
> Collect the floating film with a large spoon into a wide-mouthed bottle.
> Add an equal volume of normal sodium hydroxide.
> Shake vigorously.
> Place in incubator at 38°C. for three hours, shaking every 1/2 hour.
> Neutralize to sterile litmus with normal HCl.
> Centrifugalize.
> Inoculate sediment on Petroff's media (see p. 100).

Petroff reported that tubercle bacilli from faeces grow more slowly than do those from sputum.

EXUDATES

Exudates and transudates occur frequently in the body, the former being much more common in tuberculosis. Exudates occur in relation to an inflammatory process, are much richer in formed elements, contain serum-albumin and serum-globulin and sufficient fibrinogen to cause spontaneous clotting. They usually have a specific gravity greater than 1.018, which is the rather arbitrary level chosen as a dividing mark to separate them from transudates. The latter, on the other hand, more nearly resemble filtrates in that they are poor in cellular elements and do not clot after removal from the body. The specific gravity is usually lower than 1.018. This is the conventional description of exudates and transudates. In general, the two fluids fit sufficiently well into the characterization to be readily distinguished from each other. Exceptions are common, however, especially in the specific gravity and content of protein.

Exudates may be of various types, depending upon whether they contain large numbers of white blood cells, red blood cells, chylic or putrefactive matter. Serous or serofibrinous exudates are transparent, though rather viscid, and contain a few lymphocytes, leucocytes, red cells, large flat cells from the lining membrane, fibrin and bacteria. If pus cells (lymphocytes or leucocytes) predominate greatly, the fluid loses its transparency and becomes opalescent or opaque, whitish-yellow, and often quite thick; if red blood cells are numerous they determine the gross appearance of the fluid in proportion to their number.[3]

Exudates occur very much more commonly in the serous sacs than elsewhere and many of them have a tuberculous basis. Indeed, except for those caused by the streptococcus, exudates of these sacs caused by the tubercle bacillus greatly outnumber those having any other etiology (Maclachlan (142)). By far the majority of tuberculous exudates are serous or serofibrinous and will be discussed under the caption of serous pleural effusions. The purulent exudates are considered under empyematous effusions.

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[3] For a discussion of exudates see Webster, R. W.: Diagnostic Methods, 5th ed., Blakinston's, 1916, Philadelphia.

1. *Serous pleural effusion:* Normally there exists only sufficient pleural fluid to moisten the pleural surfaces, but in several pathological states large quantities may be quickly formed. In tuberculous pleurisy, the formation of as much as two liters may occur over night—at times to the great relief of the patient if he is suffering from acute pleuritic pain. In general, except when very large, the serous effusion is eventually absorbed spontaneously, but it tends to recur and leads to the formation of adhesions much more than if removed. The fluid in pleural effusions is generally clear and straw-colored, yet it may be cloudy or yellow and opaque, depending upon the degree of the cellular content. It is rather viscid but ordinarily flows satisfactorily through the aspirating needle. However, it is occasionally so thick that it passes through the needle slowly and with difficulty. It generally has a specific gravity of more than 1.020 and an albumin-content of usually more than 25 grams to the liter. In addition to albumin, which increases in proportion with the acuteness of the case or the rapidity of the formation of the fluid, it also has globulin. It seldom contains macroscopic blood, but, being an exudate, it does contain considerable fibrinogen and numerous cellular elements. In very freshly formed effusions, numerous polymorphonuclear leucocytes are present. The polymorphonuclears are in abundance where empyema has supervened and many have phagocytosed bacilli. In the more chronic fluids lymphocytes so predominate, however, that almost no other cell can be found. In any of the effusions red cells are detectable microscopically. The cells are counted in an ordinary counting chamber. Smears may be stained with Wright's or Wilson's or any of the other modified Romanowski stains. Occasionally crystals of uric and fatty acids, cholesterin and allied substances, fat droplets and fragmented cells are present, but these are of little practical importance. Tubercle bacilli may be found in a large percentage of pleural effusions if sufficient painstaking industry be employed. The fluid should be examined promptly after removal from the chest.

It has become almost a truism in medicine that unexplained (ideopathic) pleural effusions are tuberculous. This impression is arrived at in part from the demonstration of tubercle bacilli in the fluid and in part from the subsequent tuberculous history of persons having had pleural effusions.

This fluid is to be distinguished particularly from two others; namely, that which occurs in association with cardiac disease and that in association with pulmonary neoplasm. In cardiac disease the fluid, being a transudate, is nearly always thinner, of less cellular content and lower specific gravity than that in tuberculosis, and it usually does not clot upon standing. It tends to recur rapidly after thoracentesis and to be bilateral, and is usually associated with râles toward the bases of the lungs and other signs of cardiac disease. Occasionally a cardiac patient has blood in the sputum which might add to the difficulty of the situation, but in this circumstance the absence of tubercle bacilli from the sputum in addition to the clinical signs and symptoms of heart disease usually lead to the diagnosis without difficulty. In the case of tumor, differentiation is sometimes more difficult. As already mentioned, the fluid of tuberculous pleural effusion is generally clear and without blood, or, if sanguinous, it frequently becomes clear after a second or third thoracentesis. On the other hand, the fluid in pulmonary neoplasm is very often sanguinous, and it tends definitely to remain sanguinous and to recur. If clear at the outset, it frequently becomes sanguinous after the first few taps. The continued failure to find tubercle bacilli after careful search and the character of the symptoms, especially dyspnea, which are usually more distressing than those in simple tuberculous effusion, together with the physical signs point to the proper diagnosis.

a. *Method of obtaining:* In obtaining the fluid, ordinary thoracentesis with rigid asepsis is employed. The patient may be erect or recumbent with the back arched somewhat and the arm extended anteriorly or held above the head. Unless localization or sacculation of the fluid points to the contrary, a site for the introduction of the needle is generally selected in the middle or posterior axillary line or lateral to the inferior angle of the scapula at the level of the sixth, seventh or eighth intercostal space. A wide area of the skin in the place designed for operation should be cleaned up with iodine and this rubbed away with alcohol.

1. *Anaesthesia and thoracentesis:* Anaesthesia may be easily produced either by freezing with ethyl chloride or narcotizing with a drug, the latter giving much more comfort to the patient and much more satisfaction to the doctor. Anaesthetizing with the former is undesirable because (1) the freezing it produces does not benumb the parietal pleura which is highly sensitive, and (2) it so hardens the skin as to make it very resistant to the introduction of the needle. The latter objection is not insurmountable, however, for it may be obviated by an incision. Anaesthesia may be quickly and easily done by the injection of 1 per cent novocain, 1 per cent procain, or, possibly best of all, 1 per cent apothesine. The needle should be introduced just above the upper rim of the rib in order to avoid the intercostal vessels and nerves. It is first put into the superficial layers of the skin, and enough of the solution is injected intracutaneously to form a small bleb, after which it is removed. A moment later it is introduced into the bleb, at right angles to the skin, pushed in a short distance and a few more drops injected. After another pause it is again pushed forward into the newly narcotized tissue, when a little more of the fluid is injected. This scheme of injecting and advancing the needle is continued until the parietal pleura is reached, the latter being detected by one's tactile sense, since it is more resistant than the muscle through which the needle has just been passing. This membrane is exquisitely sensitive, but when anaesthetized in this manner it may be penetrated quite painlessly. One or 1.5 cc. of anaesthetic fluid suffices for the average thoracentesis.

If the puncture be merely exploratory, fluid may be withdrawn directly into the anaesthetizing needle. However, if thoracentesis is indicated, the aspirating needle, usually of 16 or 17 gauge and two or more inches in length, is passed along the path already made by the smaller one, either with or without a small incision having been made in the skin. Negative pressure (suction) for the withdrawal may be provided in the aspirating flask with a suction pump. Such pressure may be created also if one adds 5 cc. of 95 per cent alcohol to the aspirating flask, shakes it, applies a flame to the mouth and attaches the stopper immediately after the explosive thrust following the combustion of the alcohol. The amount of fluid in the effusion naturally varies within wide limits, but it is usually unwise to remove more than 1200 or 1500 cc. at one operation since removal of more than this frequently induces faintness, dyspnea or thoracic discomfort. Very rarely the patient suffers pleural shock during the operation. If shock should occur the needle should be immediately withdrawn and the aspiration thus quickly terminated. Aspiration may be repeated every two or three days if necessary. Fluid should be withdrawn slowly. Bottles must be new or clean and free from tubercle bacilli, living or dead (p. 3).

b. *Preparation for study:* The fluid is thus obtained by ordinary thoracentesis. If the examination is to be done immediately or within half an hour of its collection, no anticoagulant is necessary because clotting rarely occurs before that time. The fluid should be drawn into a bottle containing a solution of sodium citrate (50 cc. of a 1.4 per cent solution to the liter). Or the freshly drawn specimen heparin[4] may be added to the amount of 30 or 40 mgm. to the liter of fluid. Purulent fluids clot more slowly and frequently not at all. After a few drops have been removed for cell-count, the fluid is placed in large (100 to 200 cc.) centrifuge tubes and centrifugalized at high speed for 15 minutes. The lower 20 cc. is then taken from the bottom of each of the tubes with a volumetric pipette and deposited in smaller tubes for further centrifugalization, after which the sediment from these is again collected and the procedure repeated so that the final sediment represents a concentration of all formed elements in the entire specimen and contains all the particulate matter that was present in the original fluid. This final sediment should then be

1. examined by direct smear and stain,
2. inoculated on culture media, and
3. inoculated into at least two guinea pigs.

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[4] Heparin is prepared in physiological salt solution—0.1 gm. of the drug to 100 cc. of solution. After sterilization, it keeps indefinitely.

Other methods of demonstration of tubercle bacilli have been advocated and used, in some hands with good, in others with indifferent success. The inoscopy method devised by Jousset (93) of digesting the clot with pepsin has had considerable vogue, but the method outlined above meets the requirements in satisfactory manner and is accomplished with dispatch. If the fluid has clotted, the clot may be digested by any of the well-known methods (antiformin, sodium hydroxide, etc.). This yields bacilli in a fair percentage of instances.

2. *Empyematous effusion:* The formation of purulent effusions is not of common occurrence in early tuberculosis and only exceptionally does it appear "ideopathically" as the initial and only sign of the disease in the way an ordinary serous pleural effusion so frequently does. But its occurrence "without antecedent history of an assignable cause," especially if symptoms are mild, should point very strongly to the tubercle bacillus as the etiological factor. It does not occur with anything like the frequency of serous plural effusion. It is associated generally with a good deal of pulmonary tuberculosis and may occur either insidiously as a serous effusion that gradually becomes more and more purulent without producing many unpleasant or serious symptoms or as a fulminant empyema caused undoubtedly by ulceration of a caseous focus or (rarely) the rupture of a cavity into the pleural space. The fulminant type is associated with marked prostration, chills and fever, etc., has a grave outlook and is a surgical emergency.

Purulent exudate of this kind may be thin and only slightly opalescent or it may be very thick and of a light or cream-yellow color; on standing for several hours it sometimes separates into a thinner, clearer, upper layer and a lower, thicker layer of sediment. It may coagulate spontaneously. If newly-formed, it is alkaline, but if old or if much cellular destruction has occurred, it is acid in reaction. Older fluids contain many mononuclear or lymphocytic cells, many of them partially disintegrated and partially filled with fat droplets. In the acute empyemata there may be a preponderance of polymorphonuclear cells. There may also be a moderate preponderance of polymorphonuclears where secondary infection of the fluid has occurred, although this condition is probably much less frequently encountered than is generally supposed. In cases of secondary infection in which there is no pyopneumothorax, the contaminating microörganism is usually a staphylococcus. Indeed, it has been suggested that tuberculosis should be suspected whenever this latter germ is found in pure culture in empyematous fluid. Sometimes the discovery of other bacteria causes one to overlook the tubercle bacillus and thus leads to an erroneous diagnosis. As in other pus, there is nearly always a mass of débris, fatty acid and cholesterin crystals.

It is to be differentiated from acute non-tuberculous empyemata by the presence of the pneumococcus or other causative bacteria which are usually readily detected, by a higher polynuclear count in the latter fluids and by the history of a recent antecedent acute pulmonary disease. Empyematous fluids that are sterile when planted on ordinary media are nearly always tuberculous. Fluids in association with the so-called "meta-pneumonic" processes are sometimes exceptions which may offer serious difficulties in the diagnosis.

a. *Method of obtaining:* There is no essential difference in the manner of obtaining this and other fluids by thoracentesis (see above) except in the avoidance of infection of the needle-path. Lilienthal (131) was emphatic on this point and recommended leaving the needle in place until a syringe containing alcohol had been attached and the contents of this slowly injected along the tract as the needle is brought out. The fluid should be subjected to the action of one of the digestion procedures before it is planted on media or inoculated into animals.

3. *Ascitic fluid:* Tuberculosis of the peritoneum is looked upon by many as being of three types,—the ascitic, adhesive and ulcerative,—but such a rigid grouping is admittedly only descriptive. The more rational view contemplates these types merely as different stages of the disease. Fluid may accompany any type but is much more common in the ascitic, as the term implies, and may exist in great quantity. Indeed, the quantity may vary from a few ounces to several liters, even in comparatively small children. A rapid appearance of a large peritoneal effusion in children so strongly suggests tuberculous peritonitis

as to be almost pathognomonic (160). When formed *rapidly* it is frequently bloody. The fluid is always free in the abdominal cavity in the earlier stages but may become encapsulated later in the course of the disease.

Of viscid consistence and light amber color, the fluid bears a close resemblance to that of pleural effusion. It is very rarely purulent. It is ordinarily a little thinner and of a somewhat lower specific gravity than the latter; it contains some fibrin (so that it usually clots) and numerous cells chiefly of the lymphocytic group, although there are also present a few polymorphonuclears, particularly if the reaction has been acute. It also contains a few epithelial-like cells and numerous cells of a flat, mesothelial character as well as cellular fragments, and occasionally red blood cells. The fluids of long standing are likely to contain tubercle bacilli in noticeably fewer numbers than fresher ones. That the body destroys them by some lytic process seems likely theoretically, although no evidence of such ferments in the body has been demonstrated (114). The haemorrhagic fluids which occasionally occur in peritoneal carcinomatosis or other tumors possess essentially the same characteristics as those forming in the pleural spaces from similar cause.

Tuberculous serous effusion occasionally occurs toward the end of cirrhosis of the liver and may be confusing. The fluid in peritoneal carcinomatosis is sometimes to be differentiated from that of tuberculous peritonitis.

a. *Method of obtaining:* The fluid is obtained by paracentesis in which substantially the same technique is used as that in performing a thoracentesis. After local anesthetic and with the patient either in a sitting or semi-recumbent posture, the needle is introduced in or just lateral to the midline and caudal to the unbillicus.

Induced pneumoperitoneum, brought about by injection of air into the cavity, admits of inspection of the viscera and membranes with a cystoscope or endoscope. It has been practiced diagnostically and therapeutically to some extent in peritoneal tuberculosis, but as yet has not had very wide application.

4. *Pericardial fluid:* Tuberculosis of the pericardium occurs

with sufficient frequency to keep it out of the class of rare tuberculous affections. It is, however, more commonly revealed pathologically than clinically. Effusions must be present in amounts of at least 300 or 400 cc. to be detectable clinically and the amount may be almost unbelievably large. Removal of 2 liters at one paracentesis has been reported, and 4 liters have been found (142) at autopsy. It sometimes reaccumulates. The affection generally presents itself clinically with an effusion that may be either serous, serofibrinous, fibrinous: more rarely it is purulent. It resembles the fluid in pleural effusion so that the procedures employed in the study of the latter apply equally to it.

a. *Method of obtaining:* Lilienthal (131) suggested performing the paracentesis by introducing the needle in the third interspace near the sternum and, after very slowly passing through the thoracic integument, directing it laterally. By this means the visceral pleura remains untouched and thus uncontaminated which becomes of considerable importance if septic fluid is withdrawn. The procedure is relatively simple and should be repeated whenever fluid reaccumulates in sufficient quantity to embarrass the circulation or produce local symptoms. The introduction of air has been recommended after paracentesis has been performed, because it has been thought to aid in preventing both the rapid recurrence of fluid and the formation of adhesions by separating the surfaces (Oppenheimer (167)). If this be really true, which apparently has not yet been proved, it should be of the greatest significance.

5. *Arthritic and synovial fluids:* When arthritic fluid first develops it undoubtedly possesses the characteristics of a serous exudate, although at the time of operation, which as a rule comes a good many months after onset of the disease, it is nearly always purulent. The fluids are generally collected at operation, yet simple puncture may be employed. They may be handled in the same way as the fluids already described, but treatment with 3 per cent sodium hydroxide will facilitate the demonstration of tubercle bacilli which are rarely present in large numbers.

Pus

That the tubercle bacillus has the capacity to cause the formation of pus, alone and without the association of a so-called "secondary infection," cannot be gainsaid: witness the usual cold abscess, empyema, cervical or mesenteric adenitis, abscess of bone or kidney. That, of course, is not to say that other microörganisms may not at times be present and may not even modify the character of the exudate under question, for this undoubtedly does sometimes happen. It should be borne in mind, however, that the presence of other bacteria is not a *sine qua non* to the formation of a purulent exudate or pus, and that they are not encountered with particular frequency in such products. In this connection it should be remembered that a source of error has frequently entered when contaminating bacteria seen on culture or smear have led to a mistaken diagnosis, because they are usually much more quickly demonstrated in culture and more easily found in smear than are tubercle bacilli especially since the latter are present in pus in only small numbers.

1. *Adenitis:* Cervical adenitis is probably the most common form of clinical tuberculosis in children and is frequently enough encountered in the adult population. Aspiration of pus from these or from other tuberculous lymph nodes in different regions of the body, however, is little practiced, although this procedure has now come to be fairly common in several diseases. The reasons for this are probably two: in the first place, a large number of the abscesses are ultimately removed by the surgeon and thus the tissue and pus may be obtained; in the second place, there is the danger that an infected sinus may develop along the path of the needle. This latter issue need not follow, however, if the precautions mentioned in the consideration of thoracentesis in empyema be employed.

Pus from a tuberculous abscess of lymph nodes resembles generally that from tuberculous abscesses situated elsewhere in the body. The pus may be thick and so inspissated that it does not flow and contain little except débris and a few tubercle bacilli, or it may be thin and watery and contain only a few recognizable cells, mostly lymphocytes, and a good deal of unrecognizable matter and débris. It contains few, usually very few, tubercle bacilli, the demonstration of which frequently must depend upon inoculation of animals. Unless there is a sinus tract to the outside, other bacteria are not found.

a. *Gland puncture:* The recovery of cellular tissue by puncture, as used by Guthrie (78), is a more difficult matter than obtaining pus. Recently, however, Forkner (64) has devised a scheme which yields sufficient material for microscopic examination, regardless of whether the node is solid or liquefied. Although, strictly speaking, the method does not concern the collection of "pus" it is sufficiently germane to warrant description in this place. Forkner recommended preliminary surgical cleansing and local anaesthesia of the region and the introduction of a 17 or 18 guage one and one-half inch needle through the skin and into the node, followed by curettage through the lumen of the needle by means of a coarse, sterile dental broach with the barbs directed from the point. Such an instrument passes smoothly and neatly into tissue but upon withdrawal drags little bits of the latter on each separate barb, fish-hook fashion. Material brought in this way from the interior of the lymph node may be examined in the fresh state either by ordinary smear and stain, or by the supravital technique of Sabin (190). It may also be planted directly on culture media or in tissue culture or it may be inoculated into animals.

Characteristic tissue is recovered from tuberculous lymph nodes, according to Forkner. The predominant cell is large and oval with a small vesicular nucleus and granular cytoplasm, having a very faint yellow color. It is the ordinary epithelioid cell of tubercle. Next to this cell, lymphocytes are the most numerous. Polymorphonuclears, macrophages and numerous giant cells, as well as a deal of disintegrating débris and dead cells, help to complete the make-up of the smear. Tubercle bacilli are only infrequently found.

2. *Aural discharge:* The material from a discharging ear, usually moderately thick, yellow pus in scanty amounts, sometimes contains acid-fast bacilli. But the smegma bacillus or a closely allied type inhabits auricular secretions (cerumen, etc.)

sufficiently often to necessitate the differentiation of the acid-fast bacilli found in such secretions as between tubercle and smegma bacilli, by animal inoculation. The pus is obtained by swab or syringe or by washings from the cotton plugs that should be kept constantly in the ear during suppurative stages. Tuberculosis of the middle ear is not common and must be differentiated from chronic suppurative otitis media.

3. *Other tuberculous abscesses:* The pus from a *cold abscess, psoas* or other tuberculous abscess histologically resembles very closely that from any tuberculous abscess, in that it has a great preponderance of cellular detritus, many cells which are undergoing disintegration, and a good many normal lymphocytic and polymorphonuclear cells. The same holds true in general of the excretion from *fistula in ano.*

Cerebrospinal Fluid

1. *General properties:* In tuberculous meningitis the cerebrospinal fluid generally possesses several characteristics that indicate the probable diagnosis, before tubercle bacilli are sought. The fluid, usually under increased pressure, is limpid when first drawn, even though the count is usually above 100 cells per cu. mm. and sometimes amounts to several hundred. Occasionally in fulminating cases with a rapid course, it is slightly opalescent or turbid. A few hours after withdrawal, there nearly always develops within the fluid a faint, delicate, grayish-white pellicle or "web," which is very characteristic of tuberculous meningitis. Although characteristic, it is not pathognomonic, because a similar structure may be formed in the fluid from cases of poliomyelitis, encephalitis and perhaps other diseases. The pellicle is composed of strands of fibrin that form a delicate tracery or network in the meshes of which there collect most of the cellular elements and bacteria present in the fluid. It is therefore in this structure that one is most likely to find tubercle bacilli. The web usually extends throughout the height of the column of fluid in the tube. Of the cellular content, lymphocytes constitute 70 to 80 per cent. The cell count, however, seldom has diagnostic significance for it does not differ

materially from that in poliomyelitis, encephalitis or cerebrospinal syphilis (see Neal (160)).

Occasionally a round-cell tumor in the region of the pons sheds into the cerebrospinal fluid large numbers of cells which are indistinguishable in the counting chamber from lymphocytes and are differentiated from the latter only by staining with blood stain.[5]

There is very commonly a reduction in the amount of glucose in the fluid in tuberculous meningitis which a number of authors have claimed constitutes a pathognomonic sign, since in encephalitis, poliomyelitis and lues this substance is normal or increased. Better opinion would indicate that certainly there is nothing of a pathognomonic character about the amount of glucose present and that there are a sufficient number of exceptions to render the test of doubtful value. The amount of glucose in the cerebrospinal fluid fluctuates with the intake of food in just the same way as it does in the blood, and this fact has frequently been lost sight of. Fehling's test suffices for determining the presence or absence of glucose.

2. *Method of obtaining: preparation for study:* It requires some experience and some finesse to perform a spinal puncture, so that oversight by one with some experience is desirable until the beginner has himself perfected the technique. For doing a spinal puncture the patient is placed on his side at the edge of the bed with his legs flexed upon his body and his knees resting upon his chest. A wide area in the vicinity of the third and fourth lumbar spines is cleaned up, just as in the case of thoracentesis. After local anaesthesia, the lumbar-puncture needle, 16 or 17 gauge and 8 or 10 cm. long, is introduced, with the stylet intact, about 1 cm. lateral to the midline in the space between the third and fourth lumbar vertebrae. By a series of slight forward movements it is firmly but gently pressed inward and a little cephalward, and the stylette removed frequently to ascertain if the dura has been penetrated, the depth of this structure being dependent upon the size of the patient and the thickness of his body wall. Too great pressure may send the needle too far into the canal or may break it against one of

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[5] Personal communication from Dr. Arnold R. Rich.

the bones. If the needle is in proper position in the canal, the fluid ordinarily drips freely upon removal of the stylet and should be collected in a wassermann tube or medicine glass (thoroughly cleaned with sulphuric acid and potassium bichromate solution). No suction should ever be exerted. Not more than 10 cc. should be removed at one puncture unless special indications point to the desirability of the removal of a larger amount. Damage to the *cauda* is very uncommon. But occasionally severe headaches follow, and it is a good routine for the patient, if ambulant, to remain in bed for twenty-four hours following puncture. Fuller details of the technique may be found in textbooks of clinical microscopy such as that of Webster, Emerson, etc.

Since the pellicle contains most of the bacilli, it is to this structure that we should pay particular attention. When it has formed in a wassermann tube, its removal in the intact state is difficult. If lifted from the fluid, it immediately folds into a thread or crumples. If, while still in the fluid, it is brought in contact with a foreign object, such as a needle or forceps, it adheres with great tenacity. It loses its form when an effort is made to extricate the needle. One must carefully tilt and juggle in an effort to *pour* the web on to a slide along with a little of the fluid, there spread or float it, and then drain away the excess fluid. It is a difficult feat but it can be accomplished if sufficient time and care be employed. In case the web does become folded and distorted, it may be smeared on the slide with fairly satisfactory results. These difficulties are largely done away with if in the collecting vessel one first places a clean new cover-slip very thinly smeared with serum, then obtains the specimen and allows it to stand in the ice-box over night or for 24 hours. As a collecting vessel one may use a conical glass container, a centrifuge tube (Roby (185)) or an ordinary medicine glass. The pellicle forms directly upon the cover-slip. One then very carefully pipettes off the fluid and removes the cover-slip with small curved forceps. It is ready to be dried in the air and fixed over a gentle flame and stained.

Realizing that pellicles do not always form in spinal fluid and that certain very real mechanical difficulties at times stand in the way of detecting tubercle bacilli in such fluids, Cheer (36) has recently brought forth a new method for making the bacteriological diagnosis. By this method the cell count and differential are made, and to 5 cc. of the fluid 2 cc. of 95 per cent alcohol is added, the tube being shaken the while. If but little coagulum appears, one or two drops of egg-white are added to the fluid. The optimum coagulum is milky in appearance and resembles a "trace" of albumin in the urine after heat. The tube is then centrifugalized at fairly high speed for about one-half hour; the supernatant fluid is carefully decanted and the sediment removed and transferred to three or four slides for staining by the Ziehl-Neelsen technique. The method works satisfactorily but does not appear to be in any way superior to those described above.

Another helpful method is to add two drops of 5 per cent tannic acid to 10 cc. of the fluid to form the precipitate (14).

Blood

In tuberculosis there are no constant or typical blood changes. Although a few variations occur in the haemoglobin and blood cells, the rule is to find them remarkably near the normal line until emaciation appears.

1. *Anaemia:* The slightness of the anaemia is usually out of proportion to the pallor of the skin and physical debility associated with the disease. In chronic phthisis a slight degree of anaemia does exist but without a very noticeable fall in the red count. Whether the normal number of red cells is accounted for by blood concentration which is brought about through sweating, vomiting or diarrhoea has been questioned, but apparently these factors do not contribute materially to this condition in the rank and file of cases, and no satisfactory explanation has as yet been offered. The pallor has been attributed to the low blood pressure that is so frequently associated with the disease. The specific gravity of the blood falls, sometimes considerably. Later in the disease a true anaemia supervenes with a lowered haemoglobin and considerable poikilocytosis. Regeneration of red cells after haemorrhage occurs equally rapidly in tuberculous and non-tuberculous persons.

2. *Lymphocytes:* In tuberculosis a relative and often an actual lymphocytosis is encountered. This lymphocytic predominance, not much more than that commonly seen in states of debility or cachexia and attributed by many to this cause, is sometimes found in early stages of the disease and is nearly always a part of the picture, although without conspicuous significance. "Later in the course of the disease, especially if the progress is downward, the lymphocytic count falls somewhat."

3. *Monocytes:* There has recently developed considerable interest in the rôle of the monocyte in tuberculosis. Experimentally in rabbits, Sabin and her associates (190a) have reported a marked increase in these cells in progressive tuberculosis, but this question has not been adequately studied clinically, so that one cannot say whether the fluctuation of the monocyte has clinical significance or not.*

The term monocyte is applied to the cells formerly classified as large mononuclears and transitionals.

4. *Leucocytes:* In chronic tuberculosis there so commonly tends to be a leucopenia, that a leucocytosis points strongly against the diagnosis. In tuberculous meningitis a marked leucocytosis develops, and quite commonly in chronic tuberculosis with an exacerbation there appears a moderate increase in leucocytes, which, however, is relatively short lived. Also these cells increase where there is a great deal of absorption of necrotic tissue. The French poetically refer to the formula of resistance, the formula of defense and the formula of defeat in describing the blood picture in various types of the disease. In the first instance there is an average count of about 7,500 such as is generally found in fibroid tuberculosis; in the second instance a higher count occurs and is seen in acute phases of the disease; and in the third instance a still higher count may be found in severe types with a great deal of caseation. Such a

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*Morriss and Tan (American Review of Tuberculosis, 1927, xvi, 729) have very recently studied intensively the question of the ratio of leucocytes to monocytes in human beings with tuberculosis. They stated that a decrease in the ratio indicates a poor prognosis, and agreed with the formulae of the French which are quoted below.

grouping is subject to so many exceptions, however, that the classification itself seems more poetry than fact.

5. *Platelets:* In chronic disease in general the normal platelet count of 250,000 to 300,000 per cu. mm. is somewhat increased. The blood of the tuberculous person presents no significant changes in platelets other than a similar slight increase. But many times the count is perfectly normal.

6. *Arneth's formula:* Although introduced as a diagnostic and prognostic test, the Arneth differential formula (3) is morphological, and may properly come in for brief mention in this connection. The procedure was heralded as of great prognostic significance in tuberculosis. It consists in a differential count of the polymorphonuclear neutrophiles on a basis of the lobation of their nuclei. Several classes of these cells are given, with cells containing one, two, three, four or five lobes in the nucleus. Persons with but feeble "resistance" to the infection possess many cells containing only one or two lobes, while persons with better resistance have polymorphonuclears containing a larger number of lobes, and those doing well with the disease have a preponderance of polymorphs with four and five lobes in the nuclei. Although considerable discrepancy appears in the reports of clinical studies on Arneth's formula, it appears to have some prognostic value. But as a diagnostic procedure, it is worthless. Ordinary cover-slip blood smears stained with Wright's stain enables one to make satisfactory counts on the Arneth basis.

Disposal of Infectious Fluids, Excretions, etc., of the Body

The most satisfactory disinfectant generally is heat. Boiling for five minutes kills tubercle bacilli in sputum, but dry heat is not nearly so efficacious. When contaminated objects such as glassware, dishes, handkerchiefs, towels, etc., are to be sterilized, boiling for this length of time is adequate. Urine, faeces, effusion fluids, or pus should either be mixed with equal parts of 5 per cent carbolic acid and allowed to stand for an hour and then boiled, or they should be sterilized in the autoclave. Fluids containing a good deal of precipitable matter, such as pleural or ascitic effusion, will precipitate upon boiling, and should be poured into either a jar or beaker before sterilization in the autoclave; otherwise the precipitated matter becomes fixed in the bottle and difficult to remove.

Many disinfecting solutions will kill the tubercle bacillus but, for practical purposes, carbolic acid in a strength of 2 to 5 per cent is adequate for all needs about a laboratory. It is the cleanest and pleasantest of the disinfectant fluids. Commercial, crude carbolic acid is cheaper and more potent than c.p. phenol and is applicable to laboratory purposes, but it should not be brought in contact with the hands. Park, Williams and Krumweide (170) have given a good account of disinfectants and the means of disinfection. In chapter V of this work a discussion of the resistance of the tubercle bacillus to disinfectants will be found.

PART II

BACTERIOLOGICAL DIAGNOSIS

Chapter II

DEMONSTRATION OF THE TUBERCLE BACILLUS BY STAINING METHODS

Of the multitude of devices for the diagnosis of tuberculosis that have been elaborated both in the clinic and the laboratory, many have fallen by the wayside as useless, a good number remain and are of some utility and a few are depended upon daily for aid, but there is only one that is absolute in its relation to the diagnosis and that is the demonstration of the tubercle bacillus. The finding of the tubercle bacillus admits of no further uncertainty or equivocation in the diagnosis, but this is not to say, of course, that failure to find it (unless prolonged and repeated search be made) rules out tuberculosis because its detection is by no means a *sine qua non* for the diagnosis. Indeed the disease is doubtless diagnosed as frequently without the demonstration of the bacillus as with it. But the value of routine examination of the sputum, both when the diagnosis is clear clinically and when it is not, is so well recognized as to need no defense. If the discovery of the tubercle bacillus is so helpful a procedure in clinically recognized tuberculosis, its detection in cases where clinically the affair is not apparent must obviously have an importance scarcely to be overestimated, and the microörganism should be sought for diligently whenever its presence is suspected.

While other means are available for determining the presence of the tubercle bacillus, as will be shown later, none is so easy. rapid, or readily applicable in so large a variety of materials a, that based upon the tinctorial reactions of the bacteriums Many staining methods have been devised but only a few of the more reliable ones need be described for the purpose in hand.

Staining Methods

The detection of the tubercle bacillus in infectious material is obviously a matter of the greatest importance, and fortunately comparatively simple methods suffice for the task. Many methods have been put forward with claims for one advantage or another, and every year brings forth its new crop, but modernity in itself is not necessarily a virtue because one of the earliest methods devised yields the best results. The use of phenol as a mordant, of fuchsin as the dye and of mineral acid as the agency for decolorization, according to the scheme introduced by Ehrlich and modified by Ziehl and later by Neelsen, remains the method of choice. The Ziehl-Neelsen method will be given now and comment upon it and upon other staining methods will follow:

1. *Ziehl-Neelsen (and its evolution):* The formula recommended by Neelsen consists of:

Fuchsin (for bacilli)	1.0 gm.
Dissolved in 5% aqueous carbolic acid	100.0 cc.
To this is added absolute alcohol	10.0 cc.

Calmette (31) advised the thorough trituration of the fuchsin in the absolute alcohol which should then be poured into 5 per cent carbolic acid and thoroughly mixed. The following formula has been recommended as being easier to prepare and entirely satisfactory in results:

Alcohol 95%	100.0 cc.
Basic fuchsin	2.0 gm.

This makes a saturated solution.

Carbol fuchsin is then prepared from this solution as follows:

Saturated alcoholic solution basic fuchsin	10.0 cc.
3% carbolic acid (aq.)	90.0 cc.

Either method is satisfactory and both preparations keep for a long time.

The latter method brings the preparation of fuchsin in line with that of methylene blue, since the latter is preferably kept in stock as an alcoholic solution and is diluted and mixed when needed.

Procedure:

Fix preparation to slide with gentle heat.
Cover with carbolfuchsin stain.
Steam gently (do not boil) three minutes.
Wash in tap water.
Add acid alcohol[6] until preparation is colorless (1/2 to 1 minute).
Wash in tap water.
Counterstain with methylene blue five seconds or longer, depending upon the thickness of the smear and its mucoid nature.
Blot with filter paper.
Dry with gentle heat.
Examine.

When Koch (98) first demonstrated the tubercle bacillus, however, he did not have at hand a mixed and readily usable stain with the contrasting colors that we use today. His procedure consisted in prolonged staining with an aqueous solution of methylene blue, that had been made alkaline with a little potassium hydrate, until germ rods and all tissue-elements present in the preparation had taken on an intense blue color. The preparation was then subjected to the action of vesuvin. This dye gradually gave a brown stain to all the tissue-elements in the preparation and masked or blotted out the blue in them. It did not stain the bacilli but left them deep blue lying in the field of lighter, dirty brown.

This method, now superseded by better ones, was tedious and time consuming and not entirely reliable, and many efforts were soon made to either modify the method or to introduce new methods for demonstrating the bacillus. The first good method came only a few months after Koch's discovery and was devised by Ehrlich (54) who employed dyes with a wide range of contrast. He found that, if he treated a tissue or smear containing tubercle bacilli with a mordant (as anilin) and then stained it with fuchsin for a sufficient time, the bacilli became red along with all other structures in the preparation; that, if he then applied mineral acid, the red was removed from the tissue but not from the bacilli; finally, that, after being destained with acid, the tissue could be readily stained again with methylene blue. By such a procedure the final preparation showed brilliant red tubercle bacilli lying in a field of deep blue. Ziehl (240) substituted phenol for anilin as a mordant and used a weaker acid for decolorization. And Neelsen (161) in 1885 gave the formula the balanced proportions in which it stands today, the best of all the methods yet devised.

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[6] Acid alcohol may be prepared by adding 2½ cc. of concentrated hydrochloric acid to 97½ cc. of 95 per cent alcohol.

Nitric acid (25%) has been used for decolorization, but nitrous acid, which removes the color from tubercle bacilli, may be formed from it. If nitric acid be used, it should be first boiled to drive off whatever nitrous acid may have formed. (Personal communication from Dr. A. K. Krause.)

After a smear has been made on a slide, one may proceed in any of at least three different ways to stain the preparation. The methods are set down in the order of the dispatch or quickness with which they may be carried out. Smears should be made on the convex surface of the slide.[7]

a. *Steaming method:* This method is simple and readily performed. It consists of heating the slide over a flame after the stain has been applied. In doing this many people make use of a slide holder, but the hand is more sensitive and with the proper hold on the slide one can give it the delicate slants and tips that are required to cause the stain to cover all points of the smear during the steaming. In order to confine the stain to the smear and to keep free from stain the end of the slide designed for holding, one draws a solid line with a wax pencil across the surface of the slide, dividing it into two unequal parts, the smaller being about one inch long. The smear is then made upon the longer surface. With the thumb and index finger directed downward, the slide, having already dried in the air, is held in a horizontal plane, smear side up, and is passed two or three times through a low flame for fixation. Carbolfuchsin is then added from a dropping bottle until the smear is well covered and the slide passed slowly back and forth over a low flame so that the stain steams for three minutes. Boiling may dislodge the smear and should be avoided. Drying of the stain may cause the deposition of a precipitate that may resemble an acid-fast bacillus and is likewise to be averted by the addition of fresh stain whenever it is necessary. After the steaming has been completed, the smear is washed in tap water, destained, counterstained, dried and examined.

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[7] In the process of manufacture, slides are made with one side slightly convex, the side opposite having a corresponding concavity. One should examine the slide prior to making a smear and indicate a given side by a mark. If the smear is made on the concave side of the slide, the latter will rock upon the microscope and be unsteady.

b. *Incubator temperature method:* Another scheme which is useful when it is desirable to stain several smears at one time and get the results without delay, is to prepare and fix smears as indicated, place the slides in a staining jar containing carbolfuchsin and put this in a warming chamber, such as a paraffin oven, at about 50°C. for 20 to 30 minutes. Decolorization, washing and counterstaining are accomplished in the same manner as in the steaming. This shortens materially the time required by the cold method and gives results indistinguishable from either it or the steaming method.

c. *Cold method:* Where routine smears and stains are made, it is economical of time to use this method. In it smears are prepared and fixed in the manner outlined above and, instead of warming the stain while on the smear, the slides are placed in a tall, narrow staining jar containing carbolfuchsin and allowed to stand over night or for 24 hours, when they are washed, decolorized and counterstained. The stain should be renewed occasionally as, theoretically at least, bacilli may be dislodged or washed from smears, float in the stain, adhere to another smear and thus become a source of error.

All of these methods are equally good in producing a uniform and constant stain of the bacillus.

2. *Other methods:* Numerous methods, many of them based upon sound principle, have had more or less vogue. From these, a few of the more important are given below.

a. *Combined destaining and counterstaining (Gabbett (66)):* This is quite rapid but not satisfactory.

Stain with hot carbolfuchsin as in Ziehl-Neelsen.

Do not destain, but counterstain one-half to one minute with a solution containing the following:

Methylene blue	1.0 gm.
Sulphuric acid	20.0 cc.
Absolute alcohol	30.0 cc.
Distilled water	50.0 cc.

Wash in water.
Dry.
Examine.

This counterstaining solution decolorizes and at the same time stains blue all the structures in the preparation except the bacilli which remain red.

b. *Methyl violet* (*Much* (*157*))*:* Much has suggested several methods, all of them based on the principle employed in the Gram stain. This stain demonstrates beading (Much's granules) better than any other method and was thought by its author to detect nonacid-fast forms of the tubercle bacillus. He believed that bacilli pass through a nonacid-fast stage in their life cycle, and the granules demonstrable by this method when the Ziehl-Neelsen method showed no such structures convinced him of the superiority of his procedure. According to Böhm (20), who compared many of the staining methods, granules *are* stained by this procedure where the Ziehl-Neelsen procedure shows none, but neither bacilli nor granules are stained in specimens in which the Ziehl-Neelsen method reveals no bacilli. The Much-forms probably do not represent nonacid-fast forms of the bacillus. Much's method II is his best.

Much's method II:

Saturated alcoholic solution of methyl violet B.N.	10 cc.
2 % solution of carbolic acid	100 cc.

Add this to smear and
Heat over flame or let stand 24 to 48 hours at 37°C.
Place in Lugol's solution........1 to 5 minutes.
Expose to 5 % solution of nitric acid...1 minute.

Then to 3 % solution of hydrochloric acid....... ....10 seconds.
Wash with solution of equal parts of acetone and absolute alcohol until color disappears.
Counterstain with dilute fuchsin or with 1 % aq. sol. of saffranin (Calmette).......... ....5 to 10 sec.

c. *Reduction* (*Konrich*): The sound, basic principle of acid decolorization, while being modified from time to time, has remained highly satisfactory since the day Ehrlich first used it (1882). But Konrich (101) has employed a different principle, namely, that of staining a given preparation with carbolfuchsin and then reducing the fuchsin with sodium sulphite. That portion of the dye in the bacilli is held firmly and not affected by the reducing agent, while that in the other structures is reduced. A counterstain is then applied. Konrich's procedure (modified) is as follows:

Stain 1½ to 2 minutes with hot carbolfuchsin.
Wash with water.
Wash well with 80 % alcohol.
Decolorize with a freshly prepared 10 % solution of pure crystals of sulphite of soda for a few seconds, or at most 2 minutes[8].
Wash with water.
Counterstain ¼ to ½ minute with an aqueous solution of malachite green (50 cc. of a saturated water solution of the stain to 100 cc. of water).

In this method the fuchsin is *reduced* and not removed, and if washing in water only is done, as Konrich recommended, a part of the dye is reoxidized and the preparation again becomes red. In order to entirely avoid this, one may wash well in 80 per cent alcohol and then reduce with sodium sulphite. Very recently it has been reported by Laubenheimer (121) that dessicated sodium sulphite remains potent a longer time than ordinary sodium sulphite. This is especially so if the solution is kept at or near the neutral point to litmus.

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[8] This should be freshly prepared each day because it undergoes oxidation. A solution as old as four days is worthless.

This is a good staining method. Bacilli and other structures stain well and the contrast between the fuchsin and the counterstain is excellent. But for routine work with sputum it possesses no advantage over the widely used and satisfactory Ziehl-Neelsen or other acid-decolorization methods. The method does have points that recommend it for use in the staining of tubercle bacilli in tissues (see p. 289).

It is interesting that Ehrlich (55) in 1883 used sodium bisulphite as a decolorizing agent. He found that the preparations turned pale upon the addition of the solution but that bacilli remained perfectly stained with red. But he did not consider that the method had any advantage over his acid decolorization.

There have been at least two modifications of the Konrich technique. One of these offered by Schulte-Tigges (194), suggested the use of picric acid for malachite green as a counterstain. The other—a modification by Serkowski (196)—made the procedure a complicated one but proposed a scheme which allowed (by not staining the background) the spreading on the slide of numerous, consecutive layers of sputum each upon the prior one as soon as the earlier one had dried, and thus, as it were, made possible the staining and studying at once the equivalent of several single smear-preparations. Very little can be said as yet concerning these modifications.

d. *Precipitation* (*Cooper*): Cooper (43) has very recently suggested an innovation in staining, based on the fact that sodium chloride and several other chemical substances, when in proper amounts, precipitate the dye material from carbolfuchsin. The precipitate is formed at room temperature but disappears at 28 to 30°C. Presuming that such a deposit might occur in the body of the acid-fast bacillus when brought in contact with warm carbolfuchsin and sodium chloride, he devised the following method:

To 100 cc. of ordinary carbolfuchsin, 3 cc. of 10% sodium chloride is added.

The stain is warmed to 30°C., or above, before it is applied to the smear.

Smear is then stained as in the Ziehl-Neelsen method.

After staining, either by steaming or in the incubator, the smear is *allowed to cool before being washed and decolorized.*

In all other points the procedure is just as that in Ziehl-Neelsen staining. According to Cooper there is an increase of 5 per cent in the incidence of positive cases over that of the ordinary Ziehl-Neelsen technique when similar smears from the same specimen are examined. He claimed that more bacilli are stained in the smear and that there is much more uniformity (less beading, etc.) in the morphology of the bacilli when stained by this method.

The technique is simple and easy but has not yet had extensive trial.

TECHNIQUE FOR MAKING SMEARS: EXAMINATION OF SMEARS

1. *Sputum:* a. *Preparation and staining of smear:* 1. *The fixative:* Egg albumin, so commonly used as a fixative, absorbs and retains methylene blue and certain other stains. Serum is a better fixing reagent and may be kept satisfactorily for a long time if collected aseptically from the rabbit (or other animal), drawn into capillary tubes and these sealed at each end in a flame. Ordinarily sputum is sufficiently mucoid or albuminous to adhere to the slide without a fixative. But if it has been treated (Chapter III) fixative is required. The latter is also requisite in most of the other fluids and in all work with sections.

A small grayish-white mass in the specimen of sputum, should be removed by two spades or two hat pins, which have been flattened somewhat at the points, and transferred to the convex surface of a slide. Then, with one of the pins, the little mass of sputum is streaked back and forth across the slide time and again until the smear becomes thinned out and dry. Then the slide, smear side up, is passed three or four times through a small flame for fixation of the smear. After removal from the flame the slide should feel hot to the back of the hand but should not burn it. The preparation is then ready for the staining which may be done in any one of the ways already described.

The tubercle bacillus possesses a high percentage of waxes or lipins, and this material is very resistant to stains. Hence in the staining methods considerable time is required. More will be said later about the nature of the wax and its staining properties.

b. *Microscopic examination:* Certain *artefacts*, such as particles of food, red blood cells on edge, fragments of cholesterin from these cells, fragments of dried stain or dried sputum, small defects in the surface of the slide, inadequate destaining, or very thick smears may retain fuchsin and confuse the novice. But the shape and refractivity of such artefacts should establish their nature almost at once. There have been several reports made of acid-fast bacilli in distilled water. These, adhering to the slide, have been stained and the diagnosis of tuberculosis made. Such, fortunately, is of rare occurrence, but it should be mentioned as a possible source of mistake (Brem (22)). Specimens of sputum collected in a milk bottle are said to very occasionally (14) show milk or butter bacilli (which are acid-fast) or those collected in a vaseline bottle or smeared on an oily slide may, according to a recent report of Holman (87) contain rods which, by virtue of a coating of grease or liquid petrolatum, become acid-fast.

A sputum-preparation stained with carbolfuchsin and methylene blue is very striking under the microscope. Such a preparation is shown in figure 2. If bacilli are present, they stand out as brilliant red rods in sharp contrast to the background that is dotted about with blue staining cells—here a polynuclear, possibly containing bacilli, there a lymphocyte, in other places strands of fibrin insinuated across the field, lying between the cells or forming a basketry of blue. At times the bacilli assume a deep purple color with carbolfuchsin instead of the characteristic red. This feature has been attributed by some to the youth of the bacilli, by others to their old age, and by still others to their disintegration and decay. Some have thought it indicated a low grade virulence or a loss of virulence. The truth is, tubercle bacilli which maintain their physical integrity and retain the carbolfuchsin when properly stained according to the Ziehl-Neelsen method should be regarded as viable, virulent bacilli, be the color a bright red or dark purple. The color reaction probably indicates nothing as to virulence of bacilli. Bacilli are at times beaded and of irregular outline. Explanations of these irregularities have been offered in about the same manner as those of the color reaction but with no more justification as regards the identity, significance or virulence of the bacilli. Any assumption that either beading or irregularity in staining properties indicate a specific effect caused by the host to lessen the virulence is gratuitous (14).

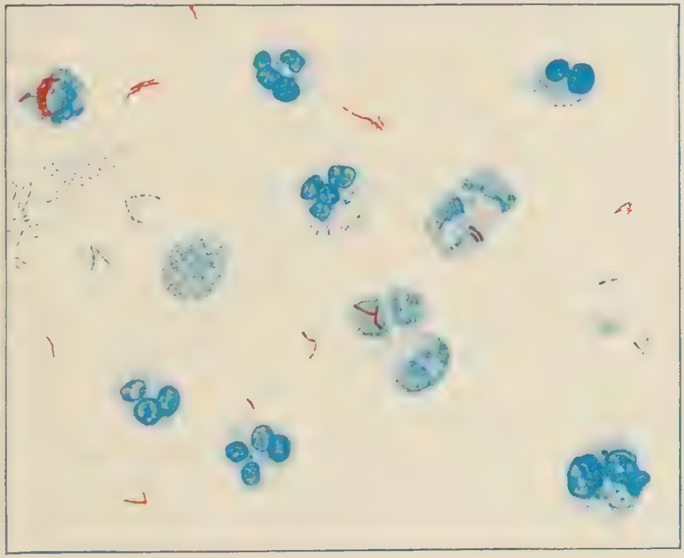

Figure 2. Color Drawing of Smear of Sputum Containing Tubercle Bacilli.

There are numerous tubercle bacilli, some contained within cells; some being extracellular. Several pus cells and cocci may also be seen. This is atypical in that polymorphs greatly predominate.

For all practical purposes the presence of acid-fast bacilli in the sputum establishes the diagnosis of tuberculosis. As has been mentioned, there do very rarely occur circumstances, however, such as in ozaena and still more rarely, in putrefactive processes (gangrene, for instance), in which acid-fast, non-pathogenic bacteria may be found in the sputum (p. 127). Such circumstances are most exceptional and the clinical picture is almost always adequate for determining the diagnosis. Specimens from such cases should be inoculated into guinea pigs.

In smears of sputum from a case of suspected tuberculosis, tubercle bacilli rank naturally as the objects of by far the greatest importance, but numerous significant structures other than bacilli are also often present and should be looked for. Most important of these are elastic tissue fibers which, if present, indicate that destruction of pulmonary tissue is going on. This tissue may be present in large or small pieces; it may remain intact in the shape of a division of a small air passage from which it came; it may be present as a flat or slightly curved plate, having come from the wall of a larger air tube; more usually it is present as short fragments or single filaments which are highly refractive, of varying lengths, usually slightly curved and always of sharp outline. If much elastic tissue is present, it is readily revealed in the smear. If it is present only in small amounts or if special search is to be made for it, a simple procedure renders its demonstration easy. This is accomplished by shaking the sputum (or tissue) in 10 per cent sodium hydrate for one-half hour and incubating at 37°C. for one to two hours. This digests most of the organic matter and leaves the elastic fibers unharmed. According to Barth, the sediment after sodium hydrate digestion may be stained for elastic fibers by subjecting it for twenty-four hours at room temperature or 5 to 10 minutes at 50 to 60°C. to a solution composed of:

Orcein	1 gm.
Water	40 cc.
Alcohol (95%)	80 cc.
Nitric acid	40 drops.

It is then washed and differentiated with a solution of 90 per cent alcohol, every 50 cc. of which contains one drop of hydrochloric acid. Elastic fibers have a brown red color after this stain has been applied.

Fatty acid crystals and vegetable spines from food are sometimes mistaken for elastic fibers. The former, however, are soluble in weak potassium hydroxide and the spines are usually recognizable by their form and shape.

Numerous bacteria besides the tubercle bacillus are generally present in the smear; predominant among them are bacteria from the mouth and throat especially streptococci, staphlyococci, diplococci, diphtheroids, micrococcus catarrhalis and others. A large number of people attribute a good deal of significance to these germs, believing that they exert a deleterious influence upon the tuberculous process in some way not clearly understood. While it is impossible for one to demonstrate absolutely the erroneousness of this position one can marshal several facts which speak against it. In the first place these bacteria are supposed to be the exciting cause of acute tuberculous pneumonia. Yet we know that the tubercle bacillus, when introduced anywhere into the body of an animal previously infected with that microörganism, will excite an acute, exudative inflammation. Besides, recent work by Austrian and Willis (8) showed that tubercle bacilli introduced intratracheally into the lungs of animals already infected with this bacterium produces pneumonia that is detectable in the roentgenogram and is typical, histologically, of tuberculous pneumonia. They could, however, produce no such response in normal, non-tuberculous animals when they employed identically similar technique. In the second place there can be no demonstrable difference in the clinical course of the patient with cavity and large numbers of "secondary" bacteria and that of the patient with cavity in which none of these bacteria were detectable. It would seem a more rational view to suppose that they are merely saprophytic.

Still other types of microörganism are sometimes present. Occasionally one of the molds (most often Aspergillus fumigatus is the cause of pulmonary disease and is found in the sputum in which the tubercle bacillus is being sought.

As first pointed out by Castellani (34), spirochaetes are occasionally encountered, usually in association with fusiform bacilli and are sometimes present in haemorrhagic sputum. It has always been supposed that these invaders are secondary to some other pulmonary injury and that spirochaetes do not produce lesion in normal tissues but may establish themselves and do much damage if they are brought in contact with injured tissues. Recently, however, Smith (200) has produced experimental pulmonary abscess by intratracheal injection of certain of these spirochaetes. The literature (Smith (199)) contains now a good many reports of these microörganisms in the sputum in persons ill with pulmonary disease, and a close relation appears to exist, in the opinion of many competent observers, between these spirochaetes and those so intimately associated with the fusiform bacillus in Vincent's angina. The disease set up by these germs *occasionally* occurs as a complication of pulmonary tuberculosis but much more often is a condition that must be differentiated from it. Pilot *et al.* (178) (179) have lately reported recovery of cases of bonchiectasis with fusiform bacilli and spirochaetes after treatment with arsphenamin. Such satisfactory therapeutic results add to the importance of these bacteria from both a diagnostic and a therapeutic standpoint.

It is unusual for these bacteria to be observed in ordinary routine preparations because the spirochaetes have usually disappeared from sputum that has stood very long, 20 to 30 minutes after expectoration sufficing for their destruction and disappearance. They should be sought in any case of chronic pulmonary infection in the sputum of which repeated examination fails to reveal tubercle bacilli. Their discovery in sputum is of sufficient importance to justify a description of the technique employed in searching for them. The patient is requested to rinse his mouth several times with water or a mouth-wash, clear his throat and expectorate and then to cough; the sputum that he coughs up is spat into a sterile petrie dish and washed through several changes of sterile physiological salt solution to eliminate bacteria from the upper respiratory tract and mouth. Smears are promptly prepared, care being taken to select small masses of pus for this purpose. They should be made slowly, the material being streaked gently back and forth across the slide because of the ease with which the spirochaetes are destroyed. They are then stained either with 10 per cent carbolfuchsin or Fontana's stain. The fusiform bacilli so con-

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stantly associated with the spirochaetes stain faintly Gram-positive. The spirochaetes are usually associated also with streptococci or other pyogenic bacteria.

The Fontana procedure (63) requires the following several solutions:

Acetic acid

Glacial acetic acid	1 cc.
Formalin	20 cc.
Distilled water	100 cc.

Carbolic acid

Carbolic acid	1 gm.
Tannic acid	5 gms.
Distilled water	100 cc.

Silver nitrate

To a 0.25% aqueous solution of silver nitrate add dilute ammonium hydrate solution drop by drop until a slight cloudiness appears.

An excess of the hydrate removes the cloudiness and is to be avoided.

This solution must be fresh.

Procedure: On the air-dried smear the acetic acid-formalin is poured, then is poured off and this is repeated several times. The preparation is next washed with water and the carbolic-tannic acid mixture added, heated until steam is formed, cooled one-half minute and again washed in water; the silver nitrate solution is next added and heated until steam forms, cooled for one-half minute, washed in water and the preparation dried and mounted.

Spirochaetes are stained brown or black. If the detection of the spirochaetes in fixed tissue is desired, the Levaditi-Manovélian (125) stain will yield the best results.

Various cells are also present. Of these, polymorphonuclear leucocytes or pus-cells in various stages of disintegration, usually predominate, sometimes to the exclusion of almost every other type of cell. Lymphocytes, red blood cells from the lungs, throat or gums, epithelial cells from the mucosa of the lungs, throat and mouth, vegetable spines and cells from food, strands of fibrin and a certain amount of débris which cannot be identified complete the picture.

c. *Variation in numbers of bacilli:* The presence of bacilli in sputum is not always a constant matter, and a failure to find them in smears from a single specimen by no means disproves the presence of tuberculosis. *Repeated daily examinations of at least fifteen minute periods should be made over an extended time in cases in which tuberculosis is suspected.* When present, their number in the sputum shows considerable fluctuation. At times the smear may contain innumerable bacilli and at other times but few—possibly none. Specimens of sputum examined daily from the same patient have been known to remain free from bacilli for weeks when, all at once, large numbers of bacilli were found. For a few days or a few weeks bacilli were still demonstrable but gradually they became fewer and finally were again absent for a considerable period. So great a variation as this is obviously encountered only unusually, but less manifest variations are continually occurring, and this fact emphasizes the necessity in suspicious cases of studying more than one smear or one specimen when this is indicated. Bacilli often completely disappear upon the onset of acute tuberculous pneumonia and may remain absent for several days or even weeks until softening or resolution occurs when they reappear in the sputum in larger numbers. They may be hard to find in sputum diluted with blood, but a careful search often reveals them.

The question occasionally arises as to whether a report should be returned as *positive* when only one tubercle bacillus has been found. In such circumstances one should obviously be certain that the slide has never been used for smears before (or if such be the case, has been treated with sulphuric acid-potassium bichromate cleaning solution) and that the wash-water was free from acid-fast bacteria (see p. 46). While one tubercle bacillus is probably sufficient for the diagnosis, it is nearly always possible to discover others by prolonged search. If none are found in the same smear, another smear should be made from the same specimen. In case no other bacilli can be found, another or repeated specimens should be examined, or several 24 hour specimens should be subjected to one of the concentration methods described in Chapter III. It is always wise to examine a second preparation where a single or even a very few bacilli have been found. Patient, painstaking and sometimes backbreaking work will nearly always be rewarded by the discovery of other bacilli in a specimen that had shown a single bacillus and will thus furnish a more certain support to the report submitted.

Gaffky (67) has suggested a scale for recording the number of bacilli in smears. An arbitrary evaluation is given to numerals so that each numeral represents an average number of bacilli to the oil immersion field. The Gaffky scale is as follows:

Gaffky 1 = 1 to 4 bacilli in entire preparation
" 2 = 1 bacillus to every few oil immersion fields
" 3 = about 1 bacillus in every field
" 4 = 2 to 3 bacilli in every field
" 5 = 4 to 6 bacilli in every field
" 6 = 7 to 8 bacilli in every field
" 7 = Numerous bacilli in every field
" 8 = Still more numerous bacilli in every field
" 9 = Very large numbers of bacilli in every field
" 10 = Enormous, uncountable, numbers of bacilli in every field.

Lawrason Brown (26) has modified the Gaffky scale as follows:

1. Only 1–4 bacilli in whole preparation
2. Only 1 bacillus on average in many fields
3. Only 1 bacillus on average in each field
4. 2–3 bacilli on average in each field
5. 4–6 bacilli on average in each field
6. 7–12 bacilli on average in each field
7. Fairly numerous on average in each field
8. Numerous on average in many fields
9. Very numerous on average in many fields
10. Enormous masses on average in many fields.

2. *Urine:* a. *Preparation and staining of smear:* The methods of collecting the urine have already been given. Irrespective of how it is obtained, the portion for examination should be centrifugalized at high speed for a minimum of 10 minutes, when the supernatant fluid is decanted and a drop of the sediment transferred to a slide and smeared, dried in the air and fixed in a flame. A small amount of serum should be spread over the slide as a fixative before the smear is made, unless the urine contains pus, in which case sufficient albuminous matter is present to act as a fixative. Walker (223) made smears on carefully cleaned slides from the sediment of the vessel and, after drying the preparation and fixing it with heat, subjected it for five minutes to the action of 5 per cent hydrochloric acid in alcohol to remove the urinary salts before staining for acid fast bacilli.

If culture or animal inoculation are desired, the sediment should first be digested with sodium hydroxide, antiformin, etc., by one of the methods outlined in Chapter III.

b. *Examination of smear:* There are few materials from the tuberculous animal that contain as many tubercle bacilli as are sometimes seen in the urine from renal tuberculosis, for at times the smear is laden with myriads of them so abundant that the entire smear retains the red color of the fuchsin after prolonged decolorization with acid alcohol. But this is not the rule, and patient, painstaking, prolonged search of several smears is sometimes necessary before bacilli are found. When they are not found after several minutes of examination, it is helpful to employ a mechanical stage so as to be more certain of examining the entire smear and as a guarantee against looking over the same area several times, as may happen otherwise. The incidence of positive results increases perceptibly when smears are examined for 10 minutes over that when the same smears are examined for only five minutes. Likewise, when 15 rather than 10 minutes are occupied with the search the incidence becomes still higher. Bacilli may be single or in small or large clumps. Numerous other bacteria may be present, especially the gonococcus and the colon bacillus, the former being easily identified by Gram's stain.

As a rule there are in the smear numerous pus cells that are frequently in a state of partial disintegration. They are predominantly lymphocytic though numerous polynuclears are present. Sometimes they are so abundant as to mask practically everything else. They may be present as granular casts in which red blood cells are sometimes intermixed. The addition of a few drops of acetic acid before staining clears the preparation a good deal. If these cells are too abundant, the sediment may be digested with sodium hydroxide which leaves only the bacilli and a little undigestible, amorphous matter. There may also be present red blood cells, the reds and whites in varying stages of preservation, flattened epithelial cells, spermatozoa, tissue fragments and débris, and gonorrheal threads.

3. *Stool:* a. *Preparation and staining of smear:* Material from the bottom of the centrifuge tube after digestion of the stool with sodium hydroxide (p. 15) is streaked across the slide with transfer spade or loop in the way described for sputum. After drying, it is fixed with gentle heat and stained by the Ziehl-Neelsen method. If the sediment is inoculated on tubes or into animals it must first be neutralized to litmus with a few drops of weak hydrochloric or other acid.

b. *Examination of smear:* Bacilli may be very numerous and in large clumps in the stool, although here, too, they may be scarce and hard to find. Their presence is an additional fact helping to make the diagnosis, but, as already intimated, their detection does not carry anything like the same significance as it does when they occur in any other of the body products. In addition to tubercle bacilli, the smear presents the undigestible products of the stool and a great deal of amorphous fragments. Smears from the fresh stool nearly always contain mucus and red blood cells.

4. *Exudates and pus:* a. *Preparation and staining of smear:* Pleural effusion, ascitic, pericardial and arthritic fluids may be described together as they closely resemble each other. The sediment remaining after centrifugalization and decantation (p. 20) is transferred to a slide with a loop or spade and streaked back and forth until dry. The slide is next passed

gently through a low flame two or three times, as for sputum, and then stained by the Ziehl-Neelsen method. If planting upon media is desired this may be readily done (p. 116), the only precaution being to continually handle the material aseptically. If the fluid be purulent, direct examination is not interfered with, but such fluid should be digested (Chapter III) before it is planted on media or inoculated into animals.

b. *Examination of smear:* The detection of the tubercle bacillus may be extremely difficult in these fluids or it may be relatively easy, especially in empyema where the germ is often very abundant. Again, the incidence is high in proportion as time and effort are expended in the quest. Between 60 and 70 per cent of pleural effusions will yield tubercle bacilli upon smear, if examinations are sufficiently thorough, and about that same figure should obtain for the other serous fluids. Tubercle bacilli are rarely very numerous or in large clumps in these fluids. Numerous lymphocytes, a few polymorphs and red blood cells and a small amount of débris complete the usual picture presented by the smear. A larger number of polymorphonuclears are present in freshly formed fluid.

The appearance of the smear prepared from purulent exudate is ordinarily that of pus and contains many partially destroyed cells of both lymphocytic and leucocytic origin, as well as many easily recognizable, more normal cells of these two types, red blood cells, both normal and degenerated, considerable débris, a few crystals of cholesterin or fatty acids, some blood pigment and a good many fat droplets. Tubercle bacilli frequently abound. It has been reported that occasionally in cases in which large numbers of tubercle bacilli could be easily found they failed to infect guinea pigs. In the more grumous fluids or more pasty types of thick pus, there is a greater proportion of unrecognizable matter, and tubercle bacilli are often scarce. If these purulent fluids are digested, only some débris and the bacilli are observable in the smear.

5. *Cerebrospinal fluid:* a. *Preparation and staining of smear:* If the pellicle has been allowed to form upon the coverslip (p. 21), it is dried in the air and passed gently through a low flame for fixation. It is then ready to be carried through

Ziehl-Neelsen staining, preferably the steaming method, and examined. When the deposit upon the coverslip is unusually heavy, decolorization is not accomplished as readily as might be expected, and it may be impossible to remove all the red stain in some of the thicker areas. It is advisable in such circumstances not to continue the decolorization too long, although prolonged exposure to acid alcohol is supposed to have no effect upon the red dye within the stained bacillus, but to counterstain with methylene blue a few moments longer than usual. Prolonged staining, destaining, washing and counterstaining may dislodge the preparation. When, on the other hand, the freshly drawn specimen is immediately centrifugalized, the supernatant fluid decanted and the sediment placed on a slide, there is as a rule so little sediment as to render it difficult to find with the microscope. It is well in this case to place the sediment on the slide, which has already been smeared with serum, in two or three drops with a pipette and allow those to dry without being smeared. After they have dried, a ring that should mark the outer limits of the drop should be made with a red wax pencil on the bottom of the slide. Such a simple manoeuver frequently aids a great deal in locating the position of the drop.

b. *Examination of smear:* Not uncommonly tubercle bacilli cannot be found in the spinal fluid although usually such fluids when put into guinea pigs produce tuberculosis. When their number is sparse, however, their detection is distinctly proportional to the amount of time and effort expended upon the search. They frequently are more abundant in the later stages of the disease so that a first specimen may fail to show them while subsequent ones readily reveal them.

Under the microscope, the web may resemble a finely meshed net composed of a great number of sinuating, intersecting threads of fibrin along which cells and bacilli lie. In those cases where smear must be made of the pellicle, the structure of the latter is greatly distorted. Cells and bacilli are found widely scattered and the latter do not tend to be clumped nearly as much as they do in urine. The cells are nearly all lymphocytes.

The fluid may be inoculated on media or into animals.

Very recently a method for detecting tubercle bacilli in spinal fluid by direct cultivation on media has been reported by Gosling and Montanus (71) from the New York City Board of Health Laboratories. The steps in the procedure follow:

Draw spinal fluid and make diagnostic smear.
Centrifugalize remainder (with pellicle if possible) for 2 hours at high speed.
Pour off supernatant fluid, leaving the bottom 1 cc. which includes the sediment.
Resuspend sediment by shaking or stirring with platinum spade.
Plant this by dropping from pipette on to slants—two Petroff's, two Lubenau's and one coagulated blood agar (the latter a control).
Seal tubes and incubate at 37.5°C.

Growth should occur in about 75 per cent of the cases and is always present 3 or 4 weeks after incubation if bacilli were in the fluid when withdrawn from the patient. Occasionally, growth by this method occurs from fluid in which bacilli could not be found in smear.

6. *Tissues:* a. *Preparation and staining of smear:* Tissues, especially lymph nodes, must occasionally be examined for evidence of tuberculosis. They usually come aseptically from operation. There is ordinarily no need for the rapid method of frozen sections in these circumstances. The technique of this procedure, however, is given on page 273. The methods ordinarily employed consist of examination of the tissue in the fresh state by smears, inoculation of media (p. 116) or guinea pigs (p. 71) and examination of histological sections (p. 284). A portion of the specimen of such tissue should be preserved in Zenker's fluid and carried on to section for histological study, but the larger portion should be studied in the fresh state. Smears should be made on slides from the cut surface of the tissue and these air-dried, fixed in heat and stained by the Ziehl-Neelsen method. Such a simple procedure is quickly done and occasionally reveals tubercle bacilli. But a more dependable way to discover hidden bacilli in such tissue consists in destroying the

anatomical integrity of the tissue by grinding it to a soft pulp in a sterile mortar containing either sand or small squares of finely woven copper gauze[9] (figure 3). To this pulp a very small amount of saline solution is added and smears are made from the thick, pasty mixture. This material may be readily planted on Dorset's egg or Petroff's media. To the remainder of the suspension, more saline solution may be added, the entire mixture poured or pipetted off into a wassermann tube and a few drops more of the saline added to the mortar as a wash. This should next be transferred into the tube which is allowed to stand for a half minute to allow the larger clumps to settle. The fluid is then drawn off down to the clumps and inoculated subcutaneously into two or more guinea pigs.

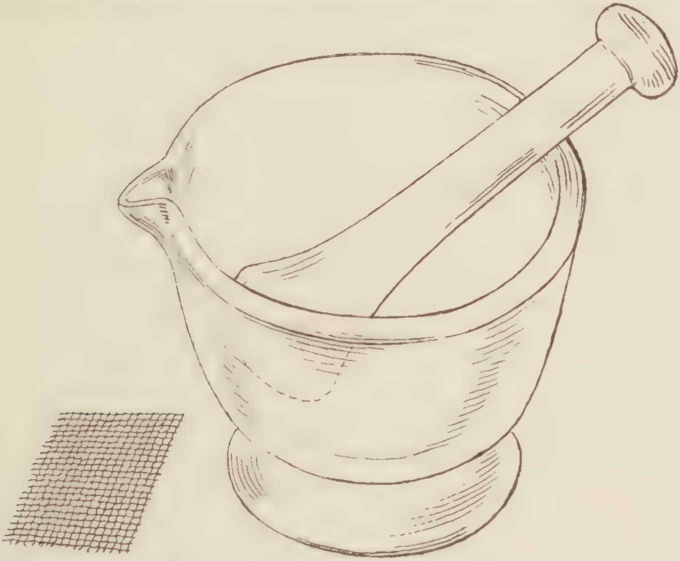

Figure 3. Mortar, Pestle and Copper Wire Gauze
Description is given in text.

Still another method may be employed especially when inoculation of tissue on to culture media is to be done. The method is described on page 228, and consists simply of crushing the tissue, e.g., bits of spleen, liver, lung or lymph node between two large sterile glass plates and planting the material directly from the plate.

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[9] Wire gauze and fine sand are equally valuable for grinding tissue.

b. *Examination of smear:* The direct smear made from the freshly cut surface will not contain many bacilli as a rule, and many times careful search fails altogether to reveal them. There will always be a few of the proper cells of the tissue, some red blood cells and a modicum of unrecognizable matter. In the smear prepared from the pulplike remains of the tissue after grinding, there is a great deal of formless stuff, a good many red blood cells and fat droplets and many of the proper cells of the tissue. Tubercle bacilli are more abundant in this material than in the smear made direct from the solid tissue, although when intermixed with so much extraneous matter, it may be difficult to find them especially when they are scarce.

7. *Blood:* Bacillaemia in tuberculosis must be a good deal more common than we are wont to think, for many of the foci of tuberculosis that are recognized clinically undoubtedly represent a haematogenous spread of the infection. Yet it has usually been a difficult matter both clinically and experimentally to discover tubercle bacilli in the blood stream (see Austrian and Hamman (7)). Smears of the centrifugalized specimen have not been fruitful in yielding tubercle bacilli, but Clough (39) has devised a scheme by which she claimed to recover bacilli in a high percentage of cases of miliary tuberculosis. Ten to 15 cc. of blood are withdrawn from arm-vein of the patient into an equal volume of 1.5 per cent solution of sodium citrate and then haemolized by the addition of distilled water, centrifugalized for one and one-half hours, some of the sediment planted on blood agar slants and some inoculated into animals. She reported a good growth usually within 10 to 14 days of the planting. Further, instead of laking the blood with distilled water, she added the blood-citrate mixture to glycerin-broth and incubated it four or five weeks. The entire culture was then centrifugalized and the sediment planted on blood agar slants or inoculated into guinea pigs. Growth on the tubes usually appeared at about four weeks after seeding. The method was applied only in a few terminal cases and should be given a more extensive trial.

CHAPTER III

DEMONSTRATION OF THE TUBERCLE BACILLUS BY METHODS OF CONCENTRATION AND ISOLATION

METHODS

As already indicated, bacilli may be present in suspected material but may be there in such small numbers as to be overlooked by smear and stain. This is especially true in early cases, and it is in such circumstances that their detection is often of the greatest value. Because of this fact numerous methods have been devised for the purpose of proving the presence or absence of tubercle bacilli in given material. A few of these will be given. They are described as they apply to sputum, for the reason that it is chiefly in the examination of sputum that they have had their application. But they may be used quite as well for any of the fluids or excretions of the body or for washings from contaminated objects, etc., the only difference being that of a variation in the duration of the digestion and a varying concentration of the digestive medium, depending upon the thickness of the material under examination.

1. *Washing method* (*Kitasato*): This is an old method (96), advanced before any of the concentration methods were put forward and is now superceded in large measure by them. The principle employed is that of washing fresh sputum to rid it of the microörganisms which adhere to it as it passes along the upper respiratory tract and the mouth and then inoculating it into animals or on media. In a sterile petri dish sputum is collected fresh from the patient, preferably after cough and after the patient has rinsed his mouth with a mouth-wash. The specimen is next transferred with two pairs of sterile forceps to the first of six or more petrie dishes containing sterile physiological saline and is sloshed back and forth several times in the fluid. It is then lifted with another set of sterile forceps

to the next dish of saline where the process is repeated. Then, each time with sterile instruments, the sputum is transferred consecutively from dish to dish and washed until the last is reached. The sputum is then inoculated into guinea pigs or planted on media. The only precaution to observe in the procedure is that of washing the specimen gently so as to avoid breaking it up.

2. *Antiformin:* Uhlenhuth and Xylander (218) in 1908 used antiformin with gratifying results as an agent to digest and homogenize sputum so that bacilli in small numbers and in thick specimens might be found. By its use the mucus, purulent material, tissue elements, and any other organic matter present in the sputum are digested, or dissolved, and the antiformin-sputum mixture becomes homogeneous. Bacilli are set free and may be recovered from the sediment after centrifugalization. Antiformin does not affect tubercle bacilli but destroys other bacteria. Brown and Smith (29) treated sputum with antiformin, inoculated the sediment on culture media and into animals and found the percentage of positive results increased over that determined by direct smear.

As recommended by Paterson (171), antiformin is prepared by mixing equal parts of a 15 per cent solution of sodium hydroxide and liquor sodae chlorinatae (U.S.P.). The latter is prepared as follows:

Sodium carbonate	600 gm.
Chlorinated lime	400 gm.
Aqua dest.	4000 cc.

(The sodium carbonate is dissolved in 1000 cc. of distilled water. The chlorinated lime is triturated thoroughly in the remainder of the water, and is filtered. The two are mixed and filtered again.[10])

The amount of antiformin used varies with the thickness or tenacity of the sputum, a stronger percentage being required in thicker specimens. The usual strength when diluted with sputum should be about 20 to 25 per cent. For dissolving the specimen of average sputum the following technique is employed:

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[10] The mixture should be prepared in a hood.

To 10 cc. sputum add 2.5 cc. of antiformin (or if a smaller amount of sputum is used, dilute to 10 cc. and add the 2.5 cc. antiformin).
Place mixture in a large (100 cc.) centrifuge tube, cork tight with an unused cork and shake vigorously.
Let stand in dark for 24 hours or in the incubator for five or six hours, during which time the tube is shaken several times.
Centrifugalize at high speed for 10 minutes.
Pipette off supernatant fluid, add physiological saline, shake, centrifugalize.
Repeat washing two more times.
Smear sediment on slides or inoculate media or animals.

All bacilli present in the 10 cc. of sputum are thus concentrated in a very small volume of sediment. Several of the reports are favorable to antiformin as a "cheap, easy and efficient method of examining sputum in which tubercle bacilli are suspected or are few in number," and from them it has been found that the antiformin method will reveal bacilli in about 5 per cent of the specimens showing no bacilli on direct smear. The method, however, is tedious, and equally good results may be got from other methods.

3. *Antiformin-culture* (*Griffith* (*75*)): This is one of the best methods we have. It is a cultural rather than a microscopic method and is rapid in the sense that bacterial demonstration can be more quickly made on cultures than by animal inoculation. It is carried out as follows:

Take 10 cc. (or less) of sputum, and
Add 10 cc. (or equal volume) of 10% antiformin.
Place in large centrifuge tube and *shake vigorously*.
Streak some of the mixture directly on solid media tubes at regular five minute intervals from five to 30 minutes (Dorset's, Petroff's). Use four or five tubes for each planting.

Only about five per cent of the tubes should show growths of contaminating bacteria and these usually occur on the set of tubes planted at the five, or that planted at the 10 minute interval. Pure cultures of tubercle bacilli may grow in any of the series of tubes, although those seeded at the 20, 25 or 30 minute intervals show more abundant growth. Lyall (140) has given a very favorable report upon this method.

4. *Sodium hydroxide* (*Petroff*)*:* This is a rapid and very satisfactory method for isolation. Petroff (176) gave the following directions:

> Place equal parts of fresh sputum and 3% sodium hydroxide in centrifuge tubes (100 cc.), cork with unused corks and shake vigorously.
>
> Incubate at 38°C. 15 to 30 minutes, depending upon consistence of the sputum.
>
> Centrifugalize at high speed for 10 minutes.
>
> Pipette off supernatant fluid.
>
> Smear sediment on slide.
>
> If culture or animal inoculation is desired, neutralize the sediment with one drop (more or less, depending upon the amount of the sediment) of 3% HCl before inoculation.

Neutralization may also be accurately done after incubation and before centrifugalization by bringing the mixture to the neutral point to litmus with hydrochloric acid.

The sodium hydroxide dissolves all the organic matter in the sputum, breaks up the thick, tenacious portions of sputum and separates the bacilli from them. In 3 per cent strength it does not kill tubercle bacilli even when incubated at 38°C. for 24 hours. It does not kill streptococci and staphylococci but has a high bactericidal titre for other bacteria. When planted on Petroff's gentian violet medium, very few contaminators grow.

5. *Hydrocarbon:* a. *Ligroin* (*Kinyoun*)*:* Kinyoun (95) has modified the Uhlenhuth method for sputum examination so

that there is a considerable saving of time and a somewhat higher per cent of positive results.

Collect sputum in a bottle that contains a 1% solution of cresol or lysol............................2 cc.
Add ligroin (sp. gravity of 0.715 or 0.720)........1 cc.
Add solution of hypochlorites (liq. sodae chlorinae, U.S.P., 100 cc.; caustic soda 7.5 gms.)....2 or 3 cc.
Shake bottle in shaker...............5 to 10 minutes.
Centrifugalize contents at moderate speed..10 minutes.

The ligroin is then at the top and directly beneath this is a soap-like layer.

Transfer a platinum loop of this soapy layer to slides.

Smear, dry and fix with heat or methyl alcohol.

Stain by the Ziehl-Neelsen method.

Examine.

The soda-lime and sodium hydroxide mixture works well. An equivalent but usually more potent solution, however, can be prepared in the laboratory according to directions given by Kinyoun. The solution made according to either formula is more potent than commercial antiformin. They remain stable for two or three months. The available chlorine is the important solvent in the mixture. The optimum concentration of this element (which should be measured) should be 5½ or 6 per cent. A solution in which the chlorine is below 3 per cent is worthless. With this method, as with the Greenfield-Anderson method (p. 66), the bacilli are usually killed and it is not to be used if cultivation or animal inoculation are desired. According to the author, bacilli are found in a search of one minute if they are found at all. Numerous laboratories use this method with satisfactory results.

b. *Chloroform* (*Andrus-MacMahon* (*1*))*:* By substituting a heavy for a light hydrocarbon and making a few other changes in the method of Kinyoun, these authors have introduced a method by which they claim to get a very great increase in the incidence and numbers of tubercle bacilli over direct smear.

The procedure follows:

Add to specimen of sputum (preferably about an ounce, but smaller quantities may be used) an equal amount of 0.5% NaOH in a 6 oz. wide-mouthed bottle that is scrupulously clean. Put in new cork. Add 2 to 4 cc. of 1% carbolic acid (aids staining).
Immerse bottle in water bath at 55°C. for 1/2 hour, shaking every 5 or 10 minutes. Insoluble particles are allowed to settle and the fluid decanted.
Cool and add 3 cc. of chloroform to 100 cc. mixture.
Shake in a mechanical shaker for 20 minutes.
Centrifugalize promptly and fairly rapidly for 20 minutes. (The chloroform by virtue of its weight carries the bacilli to the bottom of the tube).
Remove supernatant fluid and mix sediment with a spatula.
Make smears. The entire sediment may be placed on two slides, one in a thick and one in a thin smear.

Stain by the Ziehl-Neelsen technique.

The method is theoretically sound and should have a thorough try-out.

6. *Sodium carbonate* (*Greenfield-Anderson*): Greenfield and Anderson (72) have given a simple method for concentration of bacilli in sputum which may be used routinely on all specimens or upon those which are negative on simple smears.

They recommended that one prepare a solution as follows:

Sod. carbonate (crystals)	1.0 gm.
Phenol crystals	1.0 gm.
Water	100.0 cc.

Add 10 cc. of this to 5 cc. of sputum in a centrifuge tube, cover with a rubber cap. Shake for a few minutes.
Incubate at 37° C. for from 12 to 24 hours.
Centrifugalize for 15 minutes.
Pour off supernatant fluid.
Make smears from the sediment.
Stain with carbolfuchsin and examine.

The advantages claimed for the method are these:

1. It is rapid. The readiness with which bacilli are found saves more time than that required for the additional technical procedure.

2. The phenol sterilizes (usually) the sputa. No tubercle bacilli or other bacteria grow on Dorset's medium after the treatment.

3. Other microörganisms and cells remain intact and may be seen in the preparation (but nuclei do not stain). At Loomis Sanatorium positive sputum reports have increased by about 20 per cent by using the method of Greenfield and Anderson.[11]

7. *Other methods:* In 1908 Ellermann and Erlandsen (53) proposed the use of sodium carbonate followed by sodium hydroxide as a digestion-medium. The method is said to be good but time-consuming. Corper and Uyei (44) have just reported favorably on the use of sulphuric acid advocated by Loewenstein (132) for this purpose.* Still other good methods such as that of Twort (217) by which glycerine is used, avail, but those given are adequate and a longer list does not appear to be indicated.

DETECTION OF THE TUBERCLE BACILLUS IN MATERIALS OTHER THAN HUMAN BODY FLUIDS

In the laboratory one may be called upon to ascertain whether matter derived from sources other than the human body-fluids and excreta, such as contaminated objects (furniture, etc.), cow's milk, a piece of tissue, etc., contain tubercle bacilli. Simple methods for this are set down below.

1. *Contaminated objects:* Bacilli from sputum or from other material from persons with tuberculosis may contaminate any object with which they come in contact. Such objects as articles of furniture or clothing, floors, walls, side-walks, etc., may be washed with cotton swabs and physiological saline and the washings treated with equal amounts of 3 per cent sodium hy-

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[11] Personal communication from Dr. J. Stanley Woolley.

*Claims of extraordinarily satisfactory results have followed the recent publication of Hohn's modification of this method (Central bl. f. Bakteriol., 1927, Abt. I, Orig. CIII, 342). It should be widely tried.

droxide for one-half hour in the incubator, centrifugalized, neutralized, and inoculated into animals.

2. *Cow's milk:* Cow's milk is added in this connection because it must occasionally be examined and can best be discussed in this place. Hess (85) has devised a satisfactory procedure for isolating bacilli from milk which is briefly outlined below.

Specimens of market milk, kept in the ice box before examination, are well shaken and 10 cc. from each container are removed to tubes by means of a sterile pipette and centrifugalized five to seven minutes. Cream collects at the top. Two smears are made from the cream which is then removed with a sterile spoon, placed in a sterile petri dish and is diluted with a small quantity of physiological salt solution. The remaining supernatant milk is poured off to the lowest cubic centimeter in the tube, and this, containing the sediment, is saved in another sterile petri dish. Two smears are also made from this specimen. The specimens of cream and sediment may be mixed together and inoculated into two or more guinea pigs according to the method described on page 70, or each specimen may be inoculated separately into guinea pigs, depending upon whether one desires merely to demonstrate bacilli in the milk or to ascertain in which portion of it their incidence is greater. For inoculation of each specimen a different syringe and needle are used.

3. *Tissues:* Lymph nodes occasionally require diagnosis in the laboratory. This subject has been discussed in chapter II (q.v.), and in this connection it need only be said that in the event of contamination, the tissue may be ground to a pulp in a mortar and then treated for one or more hours at 37°C. with an equal amount of 3 per cent sodium hydroxide, the mixture centrifugalized, and the sediment neutralized (p. 64) and inoculated into guinea pigs or on media. The sediment should also be smeared on slides containing a thin film of serum, stained and examined for tubercle bacilli.

CHAPTER IV

DEMONSTRATION OF THE TUBERCLE BACILLUS BY INOCULATION OF ANIMALS

The idea of contagion in tuberculosis or consumption, dating back into ancient times, was very earnestly believed in by both physician and layman in the middle ages and renaissance, and, long before the time of Villemin, repeated attempts were made to prove the thesis by animal inoculation. Until his classical work (221) (222), however, none was successful. Other chapters of this book are devoted to a discussion of inoculation and care of animals, modes of inoculation and a description of the infected animal at autopsy, and the reader is referred to these for a more detailed account of these matters than will appear in this brief account, for only the bare, essential points in the inoculation and autopsy of the animal will be given at this time.

Many kinds of animals have been used for purposes of inoculation. Although the response to the introduction of tubercle bacilli undoubtedly varies a good deal in different species of animal, practically all mammals will succumb to inoculation with human or bovine bacilli when dosage is adequate. Of the many used, the guinea pig and rabbit have become by common consent the animals of choice—the guinea pig for diagnosis of tuberculosis, human or bovine, and the rabbit for differentiation between these two types. For the practical purpose which the present account seeks to meet, the guinea pig is the only animal that need be considered. This animal will almost infallibly give unmistakable evidence of tuberculosis if examined six or eight weeks after inoculation of any material containing tubercle bacilli, even though the latter have been relatively few in number.

It is one of the commonest mistakes made in laboratories to inoculate only *one* guinea pig with material for examination.

The disease evolves with relative slowness, and the animal may and sometimes does die from pneumonia or some other intercurrent infection before the lapse of this time. At least two animals should be inoculated in every case.

Care of Animals

Tuberculosis is a chronic disease, and in a tuberculosis laboratory, animals, whether used for diagnostic or experimental purposes, must be kept for a relatively long time. It is therefore very necessary that considerable care and attention be given them. Animals should go into quarantine for two weeks before they are taken into the regular animal room, in an effort to prevent the importation of epidemic pneumonia, snuffles, coccidiosis, etc. These diseases, however, sometimes occur spontaneously in the animal room in spite of quarantine. Inoculated animals should not be kept in cages with normals. Cages should be of a type that may be easily washed and sterilized, which ought to be done each week. Feeding should be done regularly twice a day: once a day, oats soaked for an hour or two, but not longer, in water, cabbage, lettuce or other like green stuff, with an abundance of hay; at the other feeding, wet oats and again an abundance of hay should be given. The hay is of value as much for bedding as for food (Chapter XIV).

Methods of Inoculation

For the purpose in hand we need consider but two methods of inoculation, namely: subcutaneous and intraperitoneal. A third, the intravenous, may be mentioned since it is the usual one for determining the type of an unknown strain.

1. *Subcutaneous:* This mode of inoculation has several advantages not possessed by the other more commonly used methods: it is the simplest, easiest, quickest, safest and is surest of results. It has the added advantage that palpation of the inoculated region will give a strong presumption, before the death of the animal, of whether or not tuberculosis is proceeding. It is simple, easy and quick in that no preliminary preparation of the animal is necessary; it is safe because an

animal can withstand a much greater dose of contaminating microörganisms under the skin than in the peritoneal cavity or in the veins; it is surest of results for the reason that a smaller number of bacilli will "take" or establish disease if inoculated subcutaneously in the region of the lymph nodes than in the peritoneum.

a. *Technique:* Either a syringe or a volumetric pipette connected with a needle as described on page 198 may be used for the inoculation. This should be made in the region of the groin, the needle entering the thin skin 7 to 10 mm. caudal to the nipple and a little lateral to the nipple line. With the animal held rigidly by the assistant, belly up, over a pan, or, if there is no assistant, with it strapped securely, belly up, on a board, the operator wets the field of operation with 2 per cent carbolic acid or other disinfectant, picks up the skin in a loose fold with one hand and with the other slips the tip of the needle through the skin into the subcutaneous space, and inoculates. Care should be taken to avoid passing the needle into the muscles of the thigh. As much as 10 cc. may be inoculated but it is decidedly preferable to use less than 5 cc. As soon as the needle is withdrawn, the area should be again washed with carbolic acid and the animal returned to the cage. When fragments of tissue too large to be injected through a needle require to be inoculated, a slit is made in the skin of the groin, the material introduced and the incision closed with a stitch.

2. *Intraperitoneal:* Many people prefer this type of inoculation. Two facts should always be borne in mind, however, when intraperitoneal inoculation is being employed: In the first place, the likelihood of producing death of the animal with other bacteria is considerable. The inoculum should be free from contaminating bacteria. Unless collected aseptically, it should be treated by one of the digestive processes, such as antiformin or sodium hydroxide, and neutralized before inoculation. Secondly, in an inoculum containing but few tubercle bacilli, the animal may escape infection. In reference to the latter statement, however, it should not be supposed that animals do not ordinarily become infected by the intraperitoneal

route, for they do. It is only an occasional occurrence when infection "misses," but it should be borne in mind.

a. *Technique:* The animal is held on its back, flat upon a pan; the unshaved skin of the belly is made thoroughly wet with 2 per cent carbolic acid or other disinfectant, is lifted up in the region of the umbilicus and the needle inserted through the elevated portion of the skin near the midline, it being directed cephalward and somewhat dorsally. If the needle is pushed in slowly, there need be no danger of puncturing the gut. When the needle is in, the inoculation is made; the needle is removed and the area again washed. No resistance to the injection is met if the needle is within the cavity, and no tumor or bleb is palpable at the site.

The chief occasion for employing intravenous inoculation arises when rabbits are to be used for inoculation of an isolated strain of bacilli in order to determine whether it be of human or bovine type, and will be called into use only uncommonly. The technique of the procedure is given in Chapter XII.

The Inoculum

When specimens are free from contamination, no digestion (p.64) is necessary before inoculation into guinea pigs. Such material as cerebro-spinal fluid or the serous fluids of pleural effusion, ascites, arthritis, etc., may be inoculated with impunity, as also may the purulent exudates from empyema or arthritis, in which no contaminating bacteria are present. But urine and faeces and usually the purulent exudates contain contaminating bacteria in sufficient quantity to cause death of the animal. These bacteria must be removed by digestion. Sputum may be inoculated with or without previous digestion, but the foreign matter in crude sputum excites considerable bubo-formation. If digestion has not been resorted to, the various fluids are centrifugalized by methods already indicated and the sediment inoculated. If digestion has been employed, the following procedures must be observed.

1. *Sputum:* In Chapter III several methods of reducing sputum to an inoculable state are dwelt upon and in this section we shall describe simply the handling of the material

from the point where it was discontinued in that discussion, that is, the actual manipulation necessary to animal inoculation.

a. *Washed:* After the last washing by this method, the remaining sputum is diluted with a little of the salt solution and inoculated directly or placed in a sterile mortar, ground and made into a suspension with physiological saline and then inoculated. Subcutaneous inoculation in the groin is preferable.

b. *Treated with antiformin:* Antiformin is irritating to animal tissues and the sediment should be passed through the washings as recommended. Then it is taken up in a small amount of salt solution and inoculated subcutaneously (p. 62).

c. *Treated with sodium hydroxide:* Sodium hydroxide likewise irritates animal tissues and should not be inoculated until neutralized. To the sediment in the centrifuge tube (Petroff method (p. 64) approximately an equal amount of 3 percent hydrochloric acid is added and the whole then taken up in a small amount of physiological saline and inoculated subcutaneously.

2. *Urine:* The directions for the preparation of urinary sediment for inoculation are given on p. 54. Urine should not ordinarily be inoculated until after treatment with antiformin, sodium hydroxide, etc., especially if it is obviously carrying pyogenic bacteria. Not infrequently guinea pigs inoculated even with clear urine are dead the morning after inoculation from either a colonic or a gas bacterium. The sediment is incubated with an equal amount of twenty per cent antiformin two to four hours, or sodium hydroxide for a half to one hour, centrifugalized, washed and inoculated.

3. *Tissues:* The microscopic demonstration of tubercle bacilli in animal tissues is often very difficult or even impossible; but, as has already been mentioned, if that tissue be inoculated —and if it contain but very few bacilli—infection of the animal will follow. For inoculation, the piece of tissue is placed in a sterile mortar, or in one which has been treated with cleaning solution[12] and washed with tap water. With the pestle it is

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[12] Cleaning solution is prepared as follows:

Potassium bichromate	75 gm.

thoroughly ground between two pieces of wire gauze or in sterile sand. Physiological saline is added and the whole is poured into a small-caliber test tube; the tissue fragments are allowed to settle for about ten seconds and then the fluid is inoculated subcutaneously into guinea pigs. Amounts as large as 10 cc. may be inoculated, but doses smaller than 5 cc. are preferable. Mortar, pestle and gauze are illustrated in figure 3.

4. *Cultures:* Bacilli may be brought into suspension for inoculation, and various means are used for standardizing dosage of bacilli. The best and easiest way for doing this is by first preparing a suspension of the bacilli in the manner described on page 236.

RESULTS OF INOCULATION

The normal evolution of the local tuberculous process following inoculation in the groin is characterized by a slow, gradual transformation of the normally soft lymph nodes into hard, shotty structures that begin to be palpable at about 10 days or two weeks after the inoculation and steadily increase in size and frequently ulcerate. The enlargement increases until, at autopsy, separate nodes are generally found to have fused and enlarged to form a mass which may measure a centimeter in its shortest diameter and may have a good deal of central caseation. The palpation of this orderly-developing, non-inflamed tumor in the groin imparts a good deal of information, during the animal's life, as to the likelihood that the inoculum contained tubercle bacilli; for it is extraordinarily unusual to observe such a change in the guinea pig from any other cause. And, although one would await autopsy before pronouncing the animal tuberculous, the appearance of the hard node just described is strong presumptive evidence of infection.

This is the sequence of events locally if a pure culture of

Water	200 cc.

Heat until dissolved and allow to cool.

Add *very slowly*, concentrated sulphuric acid	400 cc.

Stir gently but frequently.

The amount of sulphuric acid is relative. It should be added until crystals form.

tubercle bacilli is inoculated or if the inoculum contains no other pathogenic bacteria, no great amount of tissue-fragments, or no residue of digestive fluids. But in the event of any of these exceptions, large bubo is likely to form in the groin within 48 hours of the inoculation and is characterized by marked swelling, redness and heat, the structure generally proceeding to ulceration about the fifth or sixth day, after which it promptly subsides. The bubo, however, does not influence the tubercle bacilli that are present. The acute swelling has arisen and generally subsided by the time any evidence of enlargment of the nodes becomes obvious. In the presence of tubercle bacilli, tuberculosis develops alike in the animal that has bubo-formation and the one that has not.

At about six weeks after inoculation, there will have developed a picture that is characteristic, provided tubercle bacilli were in the inoculum. By this time the lymph nodes in the groin are enlarged to twice or thrice their normal size, are rubbery hard and generally have a central core of caseation. The nodes next in the line of drainage, namely, the deep inguinals and the common iliac on the side of inoculation, are enlarged by two diameters and may be firm but are often without central caseation. The spleen, the most important index of the disease in guinea pigs, is enlarged by two or more diameters, is usually soft, friable and wet, of dark, dull red color and contains from half a dozen to a very large number of small yellow or grayish-yellow areas or bosses of tubercle with a diameter that rarely exceeds 2 mm. The tuberculous areas in this organ, however, sometimes present themselves as white or white-gray bodies of irregular outline and great regularity of size which seldom is more than 1 mm. in diameter and are flush with the surface. The liver is sometimes peppered with red or reddish-yellow areas of tuberculosis smaller than a pin's head, but the rule is to find very few signs, if any, in this organ at this period of the disease. By six weeks the lungs have acquired but scant disease which may be present as a few (10 or 12) scattered, circular, gelatinous or pellucid spots of disease, the largest seldom having a diameter greater than 2 mm. Next to the spleen in importance as an index of disease stand the tracheo-bronchial lymph nodes which are practically always enlarged, lobulated, very firm, but rarely grossly caseous at this stage of the disease. When, after inoculation, the guinea pig presents a picture similar to this, the diagnosis of tuberculosis may readily be made without recourse to histological examination of the tissue (Chapter XIII).

INCREASING THE SUSCEPTIBILITY OF ANIMALS TO INFECTION WITH THE TUBERCLE BACILLUS

1. *X-Ray:* Dependence upon inoculation of guinea pigs for diagnosis has the obvious disadvantage of the relatively long time one must necessarily wait after inoculation for a report of the results. It is not surprising, therefore, that a good deal of work should have been done in an effort to render these animals more susceptible to the disease in order that the course might be rapid and the diagnosis forthcoming sooner than under normal circumstances. Very soon after the roentgen-ray came into use, several workers (Lortet and Genoud in 1896 (137), Fiorentini and Luraschi (59), Mühsam (158), and several others) exposed animals, some of them daily and rather intensively, to x-ray after infection with tubercle bacilli, but found that either no tuberculosis developed or that that which did develop was less extensive than that in control, non-exposed animals. Murphy and Ellis (159) in 1914 reported, however, that, after exposure to roentgen-rays sufficient to lower the lymphocytic count in mice, the susceptibility of these animals to bovine tuberculosis became greatly increased. Morton (156), took up this tack and exposed guinea pigs to one large dose of x-ray followed directly by inoculation of a menstrum suspected of containing tubercle bacilli. Under such circumstances he claimed that the animals became so much more susceptible that at ten days after inoculation, tuberculosis was widespread throughout the body. Kellert (94), however, was entirely unable to confirm this result, and this has been the experience of several workers who have repeated the experiment but have never reported upon it. Such discrepancies may have come about through a complicated technique and the presence of many unknown factors.

2. *Trauma:* Bloch (18) has used trauma, induced by pinching or otherwise injuring the tissues of the groin prior to inoculation, in an effort to establish a "*locis minoris resistienti*" and thus facilitate the development of tuberculosis and augment the rate of its appearance. But the net result of this as with the roentgen-ray has been that the scheme failed in the object for which it was introduced and has gone into the limbo of useless things.

Chapter V

THE TUBERCLE BACILLUS

Sources

Tuberculosis still claims a tremendous toll of human lives although organized effort, acquisition and diffusion of knowledge, and economic betterment have all operated to reduce the mortality. Much knowledge of the nature of the disease has accrued in recent decades and it is now known that people are almost never born in the infected state but that within the first 10 or 15 years of life a substantial majority acquire the infection, that by the age of 30, infection with the tubercle bacillus may, for practical purposes, be regarded as being very wide spread. This means that large numbers of people at some time during earlier life have come in contact with the tubercle bacillus and have reacted to it to form anatomic tubercle and that they possess that tubercle at the time of the reaction. For such a tuberculization of the race, the tubercle bacillus must be widely distributed in man's environment. The sources of this ubiquitous infecting agent therefore become matters of high and serious importance.

It is now well known that, although the tubercle bacillus strongly resists the cold and heat and drying effects of nature, it cannot live indefinitely outside the animal body. The ultimate sources of this infection must then be diseased man or animals and their excretions. These may be many and varied but by far the most important are human sputum and cow's milk. Urine, faeces, and pus may also be sources of infection, but proper sewage disposal and proper disposal of surgical dressings, etc., should render these of insignificant importance. It has even been claimed that the sweat may contain the tubercle bacillus, but elimination of the bacterium by this means almost certainly does not occur.

1. *Human type:* The generality of people are neither cleanly in their habits of promiscuous spitting nor sanitary in the disposal of their expectoration. The consumptive coughs and spits a good deal, and he undoubtedly deposits large numbers of tubercle bacilli on the floors of the house and on the sidewalks and streets of the cities. Many of these bacteria doubtless are killed by the sunlight; some of them on the sidewalks adhere to our shoes and are transported to the carpet and floor where the children play; and some are picked up from the street by the hands of playing children. Bacilli that are spat out with sputum become dried as the sputum is dried and pulverized and, theoretically at least, may be wafted about in the dust of the atmosphere. But actual, intimate contact with the tuberculous person and his sputum or the objects soiled by it, remains the most important source of the infection. Practically any object may be contaminated by the sputum. Clothing, objects handled or coughed upon by the consumptive, handkerchiefs, towels, books and particularly eating utensils have been found to contain tubercle bacilli (Baldwin (12)). Bacilli have been recovered from the hands of infants of tuberculous mothers, but it is extraordinarily rare to find them in mother's milk. In short, well nigh any article of furniture or clothing, the floors, etc., with which sputum may be brought into contact becomes a potential source of infection.

2. *Bovine type:* In this country infection with the bovine bacillus is not nearly as commonly encountered as it is in several parts of Europe, due to the fact of a lower incidence of the disease among our cattle. It constitutes, however, a substantial percentage of the cases of extrapulmonary tuberculosis in children. Reports from continental Europe place the amount of bovine infection at 12 to 15 per cent of all tuberculosis of childhood; those from Scotland have been found to be considerably higher than that, while in this country the best available data (N. Y. City Board of Health) place the incidence between 6 and 10 per cent of all tuberculous children. Practically the only sources of bovine bacillus are milk and butter from tuberculous cows. With pasteurization of milk becoming

more generally practiced, the incidence from these sources should be still further reduced.

3. *Avian type:* The avian tubercle bacillus appears to have been isolated from a few human beings, notably cases with renal tuberculosis. As a cause of human disease, however, it must be excessively rare and of but little clinical importance. Experiments of Fitch, Lubbehusen and Dikmans (61) have shown that hens with tuberculosis usually but not always lay eggs that are free from bacilli. The sources of this bacillus are of relatively small importance because the strain so very uncommonly affects human beings.

CHARACTERISTICS

The term *tubercle bacillus* includes only those acid-fast microörganisms that cause tuberculosis, that is, the *human*, *bovine* and *avian* types. Each of these will be described and it suffices here to say that the thread of description running through this chapter concerns primarily the human tubercle bacillus and that where differences exist between this and the other types, mention will be made of the fact. The term should never be applied to any of that host of non-pathogenic acid-fast bacteria that have been found scattered widespread in nature.

There has, however, been a renewal of interest (Ernest (58)) recently in the veterinary world in Johne's disease which is caused by an acid-fast bacillus originally thought by Johne and Frothingham (91) to be closely related to the tubercle bacillus. This acid-fast Johne's bacillus is certainly related tinctorially to the tubercle bacillus and may be also related in other ways, for it yields a product—johnin—that reacts very similarly to tuberculin. The spastic, indurative colitis which it induces in cattle appears, however, to be a disease entity, according to Ernest.

It has been recently suggested by Baldwin, Petroff and Gardner (14) that the bacillus of leprosy may be included in the list of pathogenic acid-fast bacteria since there is but little doubt as to its etiological relation to leprosy.

The tubercle bacillus is a minute form of life closely resembling the streptothrix group and having certain properties of both plant and animal life. It possesses many characteristics of interest and importance, certain of which will now be considered.

1. *Morphology:* Tubercle bacilli from cultures where they have lived a vegetative existence for some time occasionally present minor dissimilarities from those recently isolated from sputum, but these are of no significance. From whatever source, tubercle bacilli are relatively long, narrow, thin rods varying in length from 2 to 7 microns and having an average length of about 4 microns which is a little more than one-half the diameter of a red blood cell. They measure about 0.5 of a micron in thickness. Forms, frequently beaded, having from 2 to 5 or more very minute round, red staining granules in a row with no acid-fast material between and resembling short chains of streptococci may frequently be seen and have been thought by some to be transitional forms between an acid-fast and a nonacid-fast stage. Tubercle bacilli, pleomorphic, non-motile, often curved, may lie singly, in short rows or in small irregular clumps and appear at times to have branches; these latter, however, are in all probability not true branching forms but separate bacilli lying in close proximity to each other and touching at a point. The bacilli multiply by longitudinal division rather than by budding and branching, and, according to Miehe (148), division occurs about every six hours when bacilli are growing in hanging drops. Avian tubercle bacilli, irregular in shape and very inconstant in size, but usually larger than mammalian strains, do sometimes appear to have branching forms. Of the mammalian types the human tends to be longer and more slender and the bovine shorter, fatter and thicker. Such morphological differences are, however, inconstant and are usually nonexistent after a number of generations have grown on artificial media.

a. *Filterable forms:* A good deal has been said (Vaudremer (220), Arloing, et al., (2) and others) of a filterable stage (Berkefeld) through which the tubercle bacillus may pass. Filterable forms have been decribed from sputum, pus, tuberculous effusions and cultures. If this proves to be true it will, as pointed out very recently by Floyd and Herrick (62), result in a recasting of a good many of our ideas now current as to the bacteriology and epidemiology of the disease. These latter authors failed to grow bacteria when the filtrates from sputum were planted on media but they stated that they were able to find acid-fast bacilli in the tracheobronchial nodes in two of 12 guinea pigs inoculated with filtrates. The animals, however, showed no gross signs of disease. It seems altogether possible that the acid-fast rods found in the tracheobronchial nodes of these animals may be simply a few timothy bacilli that the animals may have acquired from hay. Whether such bacteria are to be found in normal, caged guinea pigs should be determined.

This is an aspect of the bacteriology of tuberculosis that will undoubtedly be investigated further within the near future. It is interesting in connection with this subject in so far as it relates to filterability of *cultures*, however, to comment on the fact that it is routine in this laboratory to always inoculate at least two guinea pigs with the filtrate obtained from the Berkefeld filter in the process of making Bouillon Filtrate. In the last 10 years a good many liters of this tuberculin have been prepared and a good many injections of it made into guinea pigs, all of which have always appeared normal when autopsied six or eight weeks after the injection. Smear and stain of the lymph nodes was not done in these instances, but, from this experience, it would appear extremely unlikely that virus with a capacity to produce tuberculosis in guinea pigs had passed through the Berkefeld filter in these instances. As yet the better opinion is decidedly against this idea of filterability.

Smears from cultures of tubercle bacilli fairly commonly reveal a few non-acid-fast bacilli. Suyenaga (214), working with what is supposed to be the original Koch strain, has shown that such forms are probably young rather than degenerated bacilli, as has been contended. He found no evidence for classifying these bacilli as a separate strain living symbiotically in the culture with the typical acid-fast rods. It is doubtful if such forms ever occur clinically in the absence of typical acid-fast forms.

2. *Types:* At the time Koch discovered the tubercle bacillus

no one entertained an idea that there was more than one type, but evidence pointing toward at least a duality in type gradually accumulated. It was found upon studying a strain of bacilli isolated by Nocard (162), (163) that the optimal temperature was higher than for other strains, and that the cultural characteristics were different, but these differences were attributed to the fact that this strain had been grown on media containing glycerine. In 1889 Rivolta (184) inoculated human tubercle bacilli into fowl where they remained many months without exciting lesions and whence they were recovered. After recovery they were found to have retained all their original virulence for guinea pigs. He also isolated bacilli from tuberculous fowl and found that they were not pathogenic for guinea pigs and suggested that there was an avian type of tubercle bacillus without virulence for certain animals—a characteristic which distinguished it from the human form. The next year Maffucci (143) and the year following Straus and Gamaleïa (211) brought forth definite proof of the separate entity of human and avian types of bacilli, each one having morphological and pathogenic identities.

In 1896 Theobald Smith (201) described a third type—the *bovine* bacillus—which also had a group of properties that placed it in a class apart from either human or avian types. Smith's bovine bacillus (202) differs from the avian on several counts and from the human, which it very closely resembles, in two important ways. These two differences center (1) about the acid curve of the media on which the bacilli have grown and (2) on the virulence of each type for rabbits. In the first case, when human bacilli are grown on liquid medium for two, three or four weeks, the reaction curve of the media, although at first losing some of its acidity, becomes more acid with the age of the culture, while that on which the bovine type is grown becomes less acid as the culture grows older (203). But these changes are not always constant.

The second and by far the best means of distinguishing the two types is the virulence test on rabbits. *Bovine tubercle bacilli characteristically kill rabbits: human tubercle bacilli usually do not.* Park and Krumwiede (169) have modified Smith's method for testing virulence in rabbits. The procedure is good. They recommended the use of rabbits preferably between 1500 and 2000 grams although the size of the animal makes no material difference. The animals are inoculated intravenously with suspensions of second or third generations of bacilli from egg medium. One group of animals (as controls) receives 1.0 mgm. and another group 0.01 mgm. of *human* bacilli intravenously. Another group of an equal number of rabbits receives 1.0 mgm. and still another group 0.01 mgm. of the newly isolated, unknown strain (intravenously). At the end of sixty days those animals that have not died are killed. The animals infected with *human* bacilli, even in large doses, seldom show tuberculosis and this, when present, is characterized by nonprogressive lesions in an organ or two. Those animals which received the doses of the untyped strain may show lesions which are comparable to those in the human bacilli controls or they may present an entirely different picture, depending upon whether the unknown strain is human or bovine, for even 0.01 mgm. of a *bovine* strain produces markedly progressive, fatal tuberculosis, many of the animals dying before the lapse of sixty days. If the unknown strain for differentiation produces marked tuberculosis in rabbits under the conditions recommended, it properly falls into the bovine group; if it fails to produce progressive tuberculosis, these conditions being observed, it is properly called a human strain.

In Cobbett's (42) experience, however, a dose of 1.0 mgm. of human bacilli is occasionally fatal to rabbits. This is also borne out by the studies of the British Royal Commission (24) in which more than one half of the rabbits receiving 1.0 mgm. of human bacilli intravenously died of tuberculosis. So the differentiation by this means is not without some difficulties. *Since 0.01 mgm. of bovine is uniformly fatal and the same dose of human bacilli is rarely, if ever, fatal, it is better to use this amount (very carefully measured) as the constant test dose.*

Ravanel (183), in 1902, gave the clinching touch to the identity of the bovine bacillus by isolating a strain of bovine bacilli from the mesenteric lymph nodes of a child that had died of tuberculous meningitis.

3. *Pathogenicity:* As is implied in the section above, there are specific differences in virulence of human, bovine and avian strains of tubercle bacilli. While Griffith (77) has isolated a number of strains of human and bovine bacilli with an attenuated virulence, the virulence of tubercle bacilli in general is remarkably stable and usually very high. Tubercle bacilli retain their native virulence for a very long time following isolation, but after many years of life on media to which it has been transferred every three four weeks, it very gradually undergoes some attenuation; so that a strain at 10, 15 or more years after its isolation, while still pathogenic and usually entirely effective in producing death of animals, requires a longer time to kill and tends to produce somewhat milder and less acute disease. Loss of virulence from this cause is apparently never complete, and passage through animals does not alter the capacity of a strain to produce disease. It is supposed that bacilli with higher virulence grow less well on artificial media than those with lower virulence, but this is not proved˙

There is one strain, however, which deserves mention in this connection. In 1891 Trudeau (215) isolated a strain from the lungs of a case of generalized miliary tuberculosis in an adult human being—a strain which was normally pathogenic for animals. For two or three years the virulence was maintained and then rather suddenly (over a period of a year or two) the strain lost its capacity to produce generalized tuberculosis and death (with a very occasional exception) in experimental animals while it retained its capacity to produce infection. Since that time it has remained unchanged. When inoculated into animals, it produces typical gross and histological reaction in the lymph nodes regional to the site of inoculation and, a little later, in the tracheobronchial lymph nodes; but gross evidence of visceral infection is almost never seen although bacilli are distributed widely over the body and may be recovered from various organs. The infection thus established is not progressive; it remains stationary for many months and slowly recedes, but probably does not entirely disappear. The animal thus infected possesses the immunity that is associated with infection, and this has led to a very great deal of valuable study on hypersensitiveness and immunity to tuberculosis.

The strain of bacilli in which Calmette and his associates (33) have produced attentuation by prolonged subculturing upon a medium containing bile is another example in which occasional loss of virulence by tubercle bacilli has been observed.

In the accompanying table (table 1) the pathogenicity of each of these three types for several different animals is given.

Table 1

Animal	*Avian*	*Bovine*	*Human*
Guinea pig	0	++++	++++
Rabbit	+	++++	+ or 0
Birds	+++	0	0
Domestic Fowl	++++	0	0
Swine	++	++++	0
Cow	0	++++	+
Sheep	0	++	+
Ape	0	++++	++++
Man	0	+++	++++*

For the guinea pig both human and bovine types are highly pathogenic; for animals such as the cow, pig and sheep the bovine type produces more virulent disease; for birds, domestic fowls and (occasionally) for rabbits the avian type is productive of disease and death; while for man and the ape human and bovine types set up fatal disease, bovine being less virulent in man than in the ape. But the conspicuous and important feature of the table (as regards typing of strains) is the reaction of human and of bovine types when inoculated into *rabbits*. These animals *respond but indifferently to human bacilli and die of disease from bovine strains.*

Grouped on the basis of susceptibility to the three types, several animals have been listed by Cobbett (42) in the manner shown in the accompanying table (table 2).

Very recently it has been claimed by Van Es and Martin (219) that bacilli resembling *avian* tubercle bacilli and patho-

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*The coincidence (very rare) of both human and bovine bacillus in the same person has been reported (Orth).

TABLE 2

Susceptibility of Certain Animals to Infection with the Three Types of Tubercle Bacili

Susceptibility to infection by the two mammalian types of tubercle bacilli.	Species of animal	Severity of disease caused by the three types of tubercle bacilli.		
		Human	Bovine	Avian
Susceptible to general infection by the bovine type the human type causing a local lesion at the seat of inoculation.	Ox, goat, cat	$\underline{\pm}$	++	0
	Pig	±	++	±
	Horse (1)	0	+	0
	Rabbit (2)	∓	+++	++
Susceptible to infection by both the bovine and the human types.	Guinea pig	+++	+++	Y
	Dog (1)	+	+	0
	Man	++	+	0
	Anthropoid apes and other monkeys	+++	+++	0
	Other wild mammals in captivity	++	++	
	Parrot and cockatoo	++	++	++
	Rat (3), mouse	+	+	+
Insusceptible to either type	Fowl and other domestic birds	0	0	++

$\underline{\pm}$ A local lesion caused at the seat of inoculation, and disseminated lesions in the lungs, sometimes fatal, after large inatravenous injections.

± Localized tuberculosis (spontaneous).

∓ Some general disease caused in exceptionally susceptible individuals after subcutaneous injection.

+, ++, +++ Various degrees of progressive tuberculosis.

Y The type of disease described by Yersin, without tubercles or necrosis.

(1) Spontaneous tuberculosis relatively uncommon. Difficult to infect artificially.

(2) Extremely easy to infect artificially, but seldom if ever tuberculous in its natural state.

(3) Spontaneous tuberculosis is rare. After intraperitoneal injection of very large doses rats succumb slowly to a general infection, which approximates to the Yersin type of disease.

genic for fowl have been repeatedly isolated from diseased cervical lymph nodes of tuberculous swine. But such bacilli appeared not to produce death of the swine. Similar observations have also been pointed to by Moore (153), Stubbs (212) and others. This bacillus has been found in a few instances to produce a septicaemia and rapid death experimentally in horses (Watson and Heath (227)) and in rabbits, death occurring at times before the formation of histological tubercle.

4. *Cultural characteristics:* Krause often says in his lectures that one of the important marks of Koch's greatness was his patience in watching his inoculated tubes and examining them day after day even when the medium had begun to dry out and shrink. The tubercle bacillus, upon whatever culture medium, grows very slowly, "leisurely," and needs oxygen, as has been shown by Novy and Soule (165). This slow growth is dry, warty and very granular; large and small islands of the cream-white, roughened material may exist side by side, may coalesce as growth continues or may remain separated from one another, as may be seen in figure 4. On liquid media the bacilli grow on the surface as a lumpy, whitish veil or crust of irregular thickness; thinner areas sometimes having a moth-eaten appearance as they surround a thicker, older layer. Even on the surface of broth, the growth appears dry and often granular. It *never grows in the liquid*, the fragments which sink to the bottom at the time of inoculation die, or at least fail to grow. It requires from 2 to 5 or 6 weeks for growth to cover the surface, depending upon strains, size of the container, and the exact reaction of the media. The film spreads over the surface and up the sides of the bottle, the fluid below remaining perfectly clear.

There are a few cultural characteristics of the human bacillus that differ from certain characteristics of the bovine or avian types but these are by no means constant or sufficiently specific to be used as the sole basis of differentiation. Human bacilli tend to grow more luxuriantly on both solid and liquid media than do bovine strains and for this reason have been called "eugonic" by the British Royal Commission (23). Human strains usually produce a canary-yellow pigment when grown on bovine serum and often a reddish pigment on potato (Griffith (77)), while the bovine strains do not produce pigment. Except for luxuriance of growth, human strains remain constant on media with or without glycerine. If grown on glycerine-free media, bovine strains maintain their characteristics for years, but, if subculturing continues for a while on glycerine broth, they become indistinguishable culturally from human strains. Bovine strains do not grow as luxuriantly as human strains (with the exception noted above of continued subculturing on glycerinated media) and have been termed "disgonic" by the British Royal Commission. Occasional strains of bovine and human bacilli, however, do not run to type culturally although they are practically always constant

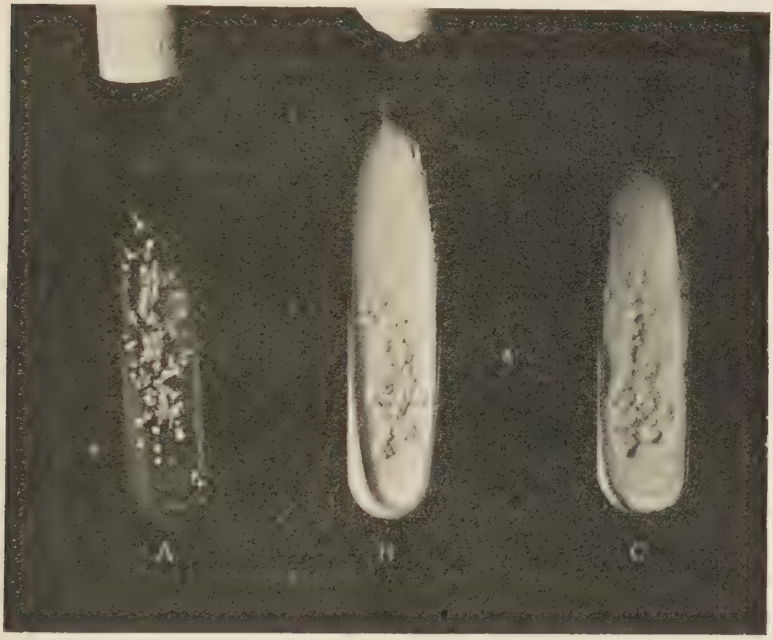

Figure 4. Photograph Illustrating Cultures of Tubercle Bacilli
Human tubercle bacilli growing on (A) glycerine agar, and (B) on Petroff's egg-beef medium. (C) represents a bovine strain on Petroff's medium.

in their relative virulence for rabbits. On solid media avian bacilli grow with relative rapidity, reaching the height of the growth at five to eight days. The growth is moist, greasy, glistening, spreading and homogeneous. Human and bovine bacilli grow best at body temperature, and grow little if at all below 35° or above 40°C. Avian bacilli, on the other hand, have an optimal temperature of about 40°C. but grow readily at 37°C.

If cultural characteristics are to be helpful criteria on which classification is made, determinations must be done before the fourth generation on media, especially if the latter is glycerinated, because bovine strains tend to adapt themselves fairly readily to test tube conditions. When isolated, bacilli should be inoculated on tubes of serum or egg, since they grow better on media that more or less resembles body fluids.

Occasionally, bacilli which have been subcultured for a long time on one kind of medium lose largely their capacity to grow. Transfer from the given medium to glycerinated potato is often efficacious in bringing back to the strain its previous growth-properties. These persist for months after the strain is planted again on the original type of medium. Prolonged transplantation on potato, however, will produce some loss of virulence.

Another peculiarity in the growth-habits of these microörganisms is seen in the difference in which subcultures grow. Ten tubes all containing media from the same batch and, therefore, all being alike as regards the pH, media ingredients, etc., may be seeded from a parent tube of good growth. On seven or eight of the tubes bacilli will grow abundantly. On the others growth often will be scanty. The cause of this is not clear although the amount of available oxygen must be an important factor (Novy and Soule (165)). But the same thing occurs with broth cultures in which cotton plugs are not sealed.

After incubator-life without transplantation for four to six months bacilli have usually lost their incubator viability but, as shown by Theobald Smith (204), these same bacilli retain the capacity to infect animals, and, upon recovery, will grow luxuriantly again on culture media.

5. *Resistance:* The characteristic resistance of tubercle bacilli to stains has been mentioned. These little bodies are also wonderfully resistant to the action of cold and heat, of physical and chemical agents, of dryness, of light. They have been found to withstand alternate freezing and thawing for months, and *dried* bacilli have remained pathogenic for guinea pigs after one and one-half years in the dark (Krause). Bacilli have the capacity to withstand for a considerable time most of the elements of nature except sunlight. And clinical tuberculosis must many times have had its source in bacilli which contaminated the surroundings and which had been deposited there months before by one sick with tuberculosis.

a. *To natural agents:* Twitchell (216) has shown that bacilli in sputum remain viable for months under circumstances in which ordinary microörganisms would be entirely unable to survive. His results were based on the capacity of tested material to initiate lesion (produce disease) in guinea pigs. In condensed form they are given in table 3. Sputa known to contain bacilli were subjected to various conditions of light and darkness,

TABLE 3

SPUTUM EXPOSED TO VARIOUS NATURAL CONDITIONS

Container	Condition of bacilli	
	alive after	dead after
Unstoppered bottles in the dark	157 days	172 days
Ice	102 days	153 days
Handkerchief	70 days	110 days
Woolen blanket	70 days	110 days
Wood (furniture?)	70 days	110 days
Carpet	39 days	70 days
Moist sand in light	123 days	148 days
Dry sand in light	30 days	70 days
Direct sunlight	1 hour	7 hours

The efficiency of sunlight naturally depends upon the altitude, cloudiness and density of the atmosphere.

time, cold, heat, drying, after having been deposited on different articles of clothing, etc. Washings from these were inoculated into guinea pigs with results as tabulated. Soparkar (206), however, found bacilli alive and virulent after sputum had been kept in the dark for 309 days, even though the specimens were long since dry. According to him, bovine bacilli are somewhat more resistant than are human bacilli. And Shope (197) reported that human and bovine tubercle bacilli, when suspended in physiological saline solution and stored in the ice chest at nearly freezing temperature for 330 days, produced typical disease and death of guinea pigs when inoculated into them. Gloyne (70) has reported that he found a tube of tubercle bacilli viable after having been six years in the ice box, most of the time, however, without ice. This culture produced a mild grade of reaction in the peritoneum of a rabbit, but the strain was not recovered and grown in culture. It is possible, therefore, that the reaction described by Gloyne may have been a response to dead bacilli. A culture of non-pathogenic acid-fast bacteria four years old, however, was found by him to grow well in culture.

Of the disinfecting capacities of the elements about us, none is nearly so effective as direct sunlight.

b. *To heat and chemicals:* Heat is an effective agent of destruction of tubercle bacilli but moist heat is much more effectual than dry heat. *Pure cultures* heated in the dry state for three hours at 158°F. have been found to retain their virulence, while moist heat kills all bacilli (cultures) at a much lower degree and in considerably shorter time. DeMan (47) found that cultures of bacilli and bacilli in milk when exposed to moist heat

At 55°C. were killed in 4 hours
" 60°C. " " " 1 hour
" 65°C. " " " 15 minutes
" 70°C. " " " 10 minutes
" 80°C. " " " 5 minutes
" 90°C. " " " 2 minutes
" 95°C. " " " 1 minute

This is significant because pasteurization heat is at the level of 65 to 70°C. If our milk is raised to this temperature for 15 minutes it should be entirely free from viable, dangerous tubercle bacilli. Bacilli in sputum are considerably protected from the ordinary disinfecting agents and a higher degree of heat must be applied for a longer period because of the mucus.

North and Park (164) have recently given the following table respecting the thermal death point of the tubercle bacillus in milk when the latter is pasteurized:

Table 4

Temperature	*Time*
212°F	10 seconds
200°F	20 seconds
180°F	20 seconds
170°F	20 seconds
160°F	20 seconds
155°F	30 seconds
150°F	2 minutes
145°F	6 minutes
142°F	10 minutes
140°F	10 minutes
138°F	20 minutes
136°F	30 minutes
134°F	40 minutes
132°F	60 minutes
130°F	60 minutes

They added, however, that, although tubercle bacilli are killed at the temperature and time of exposure indicated in the table, one should always employ a "margin of safety" that would be ample to kill all tubercle bacilli in the ordinary routine pasteurization process. Such a margin of safety is to be embraced in an exposure of 142°F. for 30 minutes.

According to Schill and Fischer (192) boiling sputum for five minutes renders it innocuous. The result of these authors' work on fresh sputum is given in table 5. After treatment, the sputum was inoculated into animals, with the results recorded in this table.

TABLE 5

Disinfecting agent	Time	Result
Dry heat 100°C	60 minutes	alive
Moist heat 100°C	15 minutes	dead
Cooked	2 minutes	alive
Cooked	5 minutes	dead
Absolute alcohol, 1 part Sputum 1 part	24 hours	alive
Absolute alcohol, 1 part Sputum, 3 parts	24 hours	sometimes alive sometimes dead
2.5% carbolic acid, 3 parts Sputum, 1 part	24 hours	alive
5% carbolic acid, 1 part Sputum, 1 part	24 hours	dead
Saturated anilin water, 5 parts Sputum, 1 part	24 hours	alive
Saturated anilin water, 10 parts Sputum, 1 part	24 hours	dead

The capacity of the tubercle bacillus from cultures to resist various chemicals has been determined. Yersin (238) has shown that *carbolic acid* in 1 per cent to 5 per cent dilution is a rapid and efficient antiseptic. Table 6 brings together Yersin's results from treatment of cultures with several disinfecting agents.

TABLE 6

Antiseptic	Living after	Dead
Carbolic acid 5%		30 seconds
Carbolic acid 1%		1 minute
Absolute alcohol		5 minutes
Ether Iodoform		5 minutes
Ether	5 minutes	10 minutes
Bichloride of Mercury 1/1000	5 minutes	10 minutes
Creosote	1 hour	
Salicylic acid	1 hour	6 hours
Thymol	2 hours	
Boric acid	12 hours	

Probable Basis of "Acid-Fastness"

The element or elements in the tubercle bacillus and the large group of "acid-fast" bacilli which is responsible for the acid-resisting capacity is not certainly known. Two dominant theories have been put forth to explain the phenomenon. The first is that of Ehrlich (55) who advanced the idea of a capsule that was only difficultly permeable. He promulgated this theory because of the resistance which bacilli offer to the penetration of stains and the equal tenacity with which they retained these dyes once penetration had occurred. This was the accepted theory until the discovery of the fact that the bacillus possesses a high percentage of fats and waxes. Then the second theory was advanced which held that the lipoidal substances were the acid-fast substances. Several workers, among them Bienstock (17), reported that they were able to effect a transformation of bacteria to acid-fastness by varying the amount of fat in the media and that nonacid-fast bacteria such as the typhoid bacillus could be rendered acid-fast by this treatment. Such work remains unconfirmed. Very recently, however, Holman (87) has shown that certain diphtheroids when brought into contact with liquid vaseline or similar oils may assume the property of acid-fastness.

Recent work has shown a very interesting relationship between the lipin and acid-fastness and has cast considerable doubt on the capsule-idea. Long (133), among others, has shown that the physical or mechanical arrangement of the lipin in the bacillary structure is responsible for the acid-fastness. From his work it appears that there are two types of lipin or two kinds of lipin-protein combination in acid-fast bacilli, the types being characterized by the resistance of the lipin to fat extractives. One of the types, comparatively loosely bound in the body of the bacillus, is readily extracted by hot alcohol and petroleum ether, and constitutes more than 20 per cent of the body weight of the dry bacilli. After such treatment bacilli retain their form and their acid-fastness. If such bacilli are then dried and treated with normal hydrochloric acid at 37°C. for forty-eight hours and again extracted with ether, lipin is yielded in amounts that equal about 4 per cent of the dry weight of the bacilli, and the bacilli after this second extraction lose their form and their acid-fastness. The lipin is itself acid-fast, and is chemically like the more easily extracted lipin. It may be bound physically to the protein of the bacillus. Long also reemphasized the fact that mechanical grinding of bacilli does away with their acid-fastness.

It appears that the *physical combination or physical arrangement of a portion of the lipin and the other elements which go to make up the bacillus is in some way related intimately to the property of acid-fastness.* This is supported by three facts: (1) An intimate relation exists between lipin and bacillary structure; (2) the nonacid-fast bacterium, B. subtilis, and possibly others, yields approximately the same percentage of lipin upon acid treatment as does the tubercle bacillus upon second extraction, yet is nonacid-fast; (3) dried acid-fast bacilli, when completely pulverized by grinding, lose entirely their acid-fastness, the amorphous residue having no acid-resisting property left.

In the past, numerous authors have assigned several important attributes to the property of acid-fastness, among them the immune responses of the body to the bacillus, the virulence of the bacillus and its resistance to the body's efforts at destroying it and to external influences, etc. But none of these theses has been proved.

CHAPTER VI

CULTURE MEDIA AND THE CULTIVATION OF THE TUBERCLE BACILLUS

Most pathogenic bacteria require that the nutrient matter upon which they grow shall approximate more or less closely the complex content of the animal tissues upon which they feed in the body, and this must be borne in mind in the preparation of media. Substances, therefore, such as meat, peptone or serum, egg, glycerine, various carbohydrates and some of the mineral salts are employed as ingredients of media. These substances are modified and chemically changed somewhat by the necessary heating incident to sterilization, but the alkaline-acid reaction may be adjusted so that most bacteria grow satisfactorily. (For a discussion of culture media in general see Park, Williams and Krumwiede (170) or other standard work on bacteriology.)

Tubercle bacilli grow slowly. They grow well upon favorable media, but poorly or not at all upon media which are less favorable but which might very well furnish the necessary ingredients for the growth of many other bacteria. When bacilli grow on one medium for a long time, that is, for 15 or more transplant generations extending over a period of a year or longer, growth sometimes gradually becomes less vigorous until finally transplants will cease to grow altogether. But, at this juncture, if transplants are made to another type of medium, growth that was slackening again becomes abundant. For instance, bacilli, which have been subcultured for many months on glycerine agar and have finally come to grow very poorly, may be transferred to glycerinated potato where they grow in very satisfactory manner. Upon return within a few weeks to glycerine agar again they grow abundantly and will continue to do so for many months. It appears that by a brief and occasional residence upon a medium with which they are not acquainted, especially potato, tubercle bacilli acquire some ingredient necessary to abundant, luxuriant growth, which they do not get after continued existence upon one type of medium.

METHODS

1. *Preparation of glassware:* Before entering upon a consideration of the making of media, a word should be said of the preparation of the tubes and other glassware which are to contain the media. Glassware as it comes from the manufacturer contains traces of alkali and sometimes spores of various bacteria. New tubes should therefore be allowed to stand over night in a weak (about 2 per cent) solution of hydrochloric acid. They are then washed in running tap water and placed in distilled water for 24 hours after which they are allowed to drain and dry at room temperature. Old tubes, in which cultures remain, should be sterilized in an autoclave, the bulk of the old media removed, and the tubes heated over a low flame in soapy water. They then should be cleaned with a brush and hot tap water and soaked in a 2 per cent solution of hydrochloric acid as new tubes are; this should be followed by washing in tap water, and soaking for 24 hours in distilled water. After the tubes are dried, raw cotton plugs (absorbent cotton takes up water too freely) are folded, rolled and placed in the mouth of the tubes to a depth of about 2 cm. Some recommend that the cotton plugs be placed tightly enough to just remain in the tube when the latter is lifted from the table by the plug. After plugging, the tubes are placed in a dry sterilizer and heated to 160°C. for one hour. This both sterilizes the tube and "sets" the plug to it which greatly facilitates subsequent handling. The tubes are then ready for receiving media. Thick, heavy-walled test tubes (culture tubes) 16 x 150 mm. without a lip are most satisfactory.

Large flat 8 to 12 oz. bottles may be used to good advantage when liquid cultures are desired. Bottles, old or new, should be prepared according to the procedure outlined above for the treatment of test tubes.

2. *Preparation of media:* Because of the slow growth of tubercle bacilli, the drying of the media was a very real problem in the early days. In 1885 Nocard (162) added glycerine in 4 or 5 per cent strength to his media in order to prevent this disastrous occurrence. To his great surprise, not only was the media saved from rapid drying but the bacilli on glycerinated media grew much more quickly and luxuriantly than before this addition. So the use of glycerine, a very valuable ingredient in media, was stumbled upon by accident. With the routine sealing of culture tubes, glycerine is no longer necessary to prevent drying of media, but it is used in nearly all media designed for the cultivation of tubercle bacilli because it greatly enhances such growth (except in first plants of newly isolated bovine bacilli).

Chemical change occurs with increase of acid in media if the latter is allowed to stand unused for any considerable time (three months or so), and large quantities of media should not be put in tubes at the time of making unless it is all to be used within a few weeks. Media may be kept in bulk in flasks for a long time, however, for, upon retitration or proper adjustment of the pH, it again possesses all the requisite elements favorable to vigorous bacillary growth. Media, whether in tubes or flasks should be kept in a cool, dark place until used.

Tubercle bacilli grow on either solid or liquid media. Where they are wanted in large quantities or where tuberculin is to be prepared, they should be grown on liquid media in large bottles since on this type of medium, growth is more rapid and can be accomplished in bulk and the broth itself may be utilized for making tuberculin. For isolation of tubercle bacilli, solid media only can be used. The latter is also highly useful for ordinary routine laboratory work. Literally scores of media have been described and used but we shall consider here formulae and directions for making only a few of the best known and most satisfactory of these.

a. *Solid media:* Routine transferring of bacilli for several years on various artificial media has convinced me that for general, all around purposes glycerine agar and egg-beef (Petroff) are probably the best solid media we have. The latter is a combination medium containing ingredients used in several good media. It is prepared according to the following formula (176):

1. *Egg-beef (Petroff):*

I. Meat Juice:

Lean beef (or veal)	500 gms.
Water	425 cc.
Glycerine	75 cc.

Chop or grind the meat and infuse in the glycerine water for 24 hours in the ice box, squeeze in gauze and collect juice in a sterile beaker.

Instead of the meat juice, 10% glycerine broth may be used.

II. Eggs:

Sterilize one dozen by immersing in 70% alcohol for 10 minutes or by pouring hot water over them, break into a sterile beaker, beat well, filter through sterile gauze. Or filtering may be delayed until after the addition of the meat juice at which time the mixture is more easily filtered because it is thinner.

To one part of meat juice add two parts of egg

Tube

Inspissate in Arnold sterilizer three successive days at 75° to 90°C.

Petroff suggested a third part to the formula which consists of the addition of a 10 per cent alcoholic solution of gentian violet to make a proportion of 1 to 10,000. This is done to inhibit the growth of other microörganisms. But in our experience the addition of the dye is usually unnecessary.

For tubing this or any other solid medium, a six-inch funnel is connected to a rubber tube three or four inches long, the free end of which is fitted over a small glass tube with a slight constriction at its end. A pinchcock is placed on the rubber and the system set up in a vertical position in a ring stand. Medium is poured into the funnel and one tube at a time is filled by placing the free end of the glass tube into the culture tube and with the pinch-cock allowing about 5 cc. of medium to flow into the latter. The entire apparatus must be sterile. The system may be properly connected and then sterilized by steam (not under pressure) in an autoclave. It is desirable to keep the funnel loosely covered while it contains the medium.

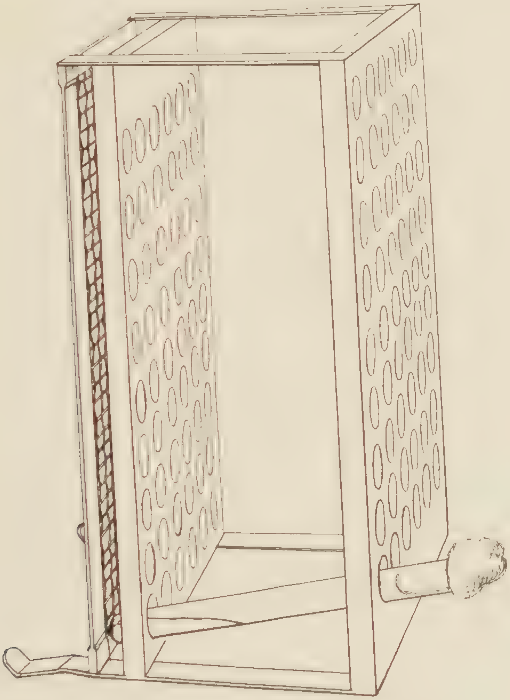

Figure 5. LINE DRAWING OF INSPISSATING RACK

Tubes of egg, egg-beef or serum are placed in the rack, which may then be transferred to an Arnold sterilizer for inspissation and sterilization. The liberal amount of open space about each tube allows uniform heating throughout. The slant of the holes causes the fresh medium to come to that position in the tube for hardening which will give a wide surface for planting.

Either an inspissator or an Arnold sterilizer may be used for sterilizing solid media. With use of the Arnold sterilizer, it is well to place the tubes in a rack such as that shown in figure 5, at the proper inclination for the slant, for several of these media are hardened into their final shape by heat. The tubes are heated to 70° or 75°C. for an hour on each of three successive days.

2. *Glycerinated agar:* Except for isolation of new strains, this is an excellent medium for growing tubercle bacilli in pure culture. First plants grow very poorly upon it, however.

The requisite ingredients are:

Lean beef	500 gm.
Peptone	10 gm.
Sodium chloride	5 gm.
Agar	20 gm.
Water	1000 cc.
Glycerine	50 cc.

Soak the beef, ground finely, in the water for 18 to 24 hours in the ice box.

Boil for 45 minutes.

Strain through six layers of cheese cloth.

Gradually add agar, peptone and sodium chloride, keeping the solution hot meantime. Stir frequently,[13] until agar melts.

Add water q.s. to offset evaporation.....1000 cc.

Filter through folded filter paper. This is best done by placing filter and receiving flask in an Arnold sterilizer which is kept at a temperature of about 70°C.

Filtration may also be satisfactorily done through a Buchner funnel. Over the perforated bottom, place three thin layers of ordinary absorbent cotton so that the grain or strands run at right angles to each other and connect the funnel to a suitable suction apparatus.

Titrate (phenolphthalein) to 1.5 acid N/20 NaOH (p. 109) or correct acidity to pH 6.8 (bromthymol blue) (p. 113).

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[13] Considerable time is required to dissolve the agar; to prevent great evaporation, one places the entire mixture in a large flask and boils it in the Arnold sterilizer, shaking the flask frequently instead of stirring.

Add glycerine .... 50 cc.
Tube
Autoclave 30 minutes at 15 pounds pressure.

If all the material is not to be tubed at once, it is advisable to separate the portion to be tubed from the other and add glycerine only to that portion which is to be used first. The untubed portion is autoclaved in a flask and kept in a dark closet until such time as it is needed: then it is melted in an Arnold sterilizer, is retitrated and glycerine is added to make 5 per cent strength. Then it is placed in tubes and autoclaved.

Some add egg (two to the liter of material) for clearing, but medium prepared as outlined is nicely transparent, and the addition of the egg seems unnecessary. After removal from the autoclave, the tubes are placed on an incline on the table so that the liquid flows in such manner in the tube as to form a long, slanting surface. In this position it hardens.

3. *Dorset's egg:* Since its introduction in 1902 (50) this medium has been extensively used for isolation of tubercle bacilli. Peculiarly adapted for nourishment of tubercle bacilli in their early sojourn out of the animal body, it is used widely for direct planting from animal tissues or from antiformin or other isolation media. Simplified somewhat, it is as follows:

Six fresh eggs are washed with alcohol and allowed to dry. They are then broken carefully and their contents dropped into a wide-mouthed, sterile flask. This is shaken gently to produce a thorough mixture but not vigorously enough to produce foaming of the egg white. Twenty-five cc. of sterile water is added to each six eggs. The mixture is then tubed, 5 cc. being placed in each tube. These are put in a rack (figure 5) and inspissated at 70° in an Arnold sterilizer or in an inspissator for 4 or 5 hours on each of three successive days. Higher temperatures do no harm. Lubenau (138) has suggested the addition of glycerine instead of water to the eggs and stated that bacilli grow better on such a medium.

4. *Glycerinated potato:* This medium, introduced by Pawlowsky (173) in 1888, is especially useful for rejuvenating strains of tubercle bacilli which have, by prolonged subculturing

upon a given medium, gradually lost their original capacity for good growth. Prolonged growth upon potato, however, causes some diminution in virulence. It is prepared as follows:

Cylinders of large, clean, peeled white potato are cut with a cork borer and are then, by a diagonal cut, divided into two wedge-shaped pieces, the thin ends of which should be left thick enough to prevent curling. The diagonal, flat surface should be 4 or 5 cm. long and smooth, for it is upon this surface that subsequent planting of bacilli is to take place. These pieces are soaked in running water over night (potatoes are acid in reaction) or, better, in a 1 to 1,000 solution of sodium carbonate. They are then drained and placed in a 5 per cent solution of glycerine for 24 hours, drained again and placed, butt end down and along with a few drops of glycerinated water, in test tubes and autoclaved for one-half hour at 15 pounds pressure. If ordinary test tubes are used, some form of support such as a small piece of glass rod must be dropped into the tube first. This holds the potato out of the fluid and prevents its becoming too wet. A better scheme is one recommended by Roux (189) of using a tube with a slight constriction in the lumen about 1 cm. from the bottom which leaves a bulbous end. The constriction supports the potato when a short distance above the fluid. Figure 6 illustrates both types of support.

5. *Potato-bile:* Calmette and Guérin (32) claimed that by the addition of bile to media they could determine whether a bacillus is of human or bovine origin and also that the original virulence of a bacillus can be greatly reduced by growing it upon potato that has been impregnated with glycerine and bile. Apparently cultures do lose in virulence when grown for a long time upon this medium. But it is open to question, however, whether prolonged transplantation to potato medium will not produce the same result without the bile.

In preparing the medium, the contents of several fresh gall bladders is autoclaved in a sterilized flask and allowed to stand at room temperature for three weeks, when it is filtered through paper. Glycerine is then added to 5 per cent, and pieces of potato immersed in this for three hours in a water bath at 75°C. The pieces of potato are then drained, placed in tubes and

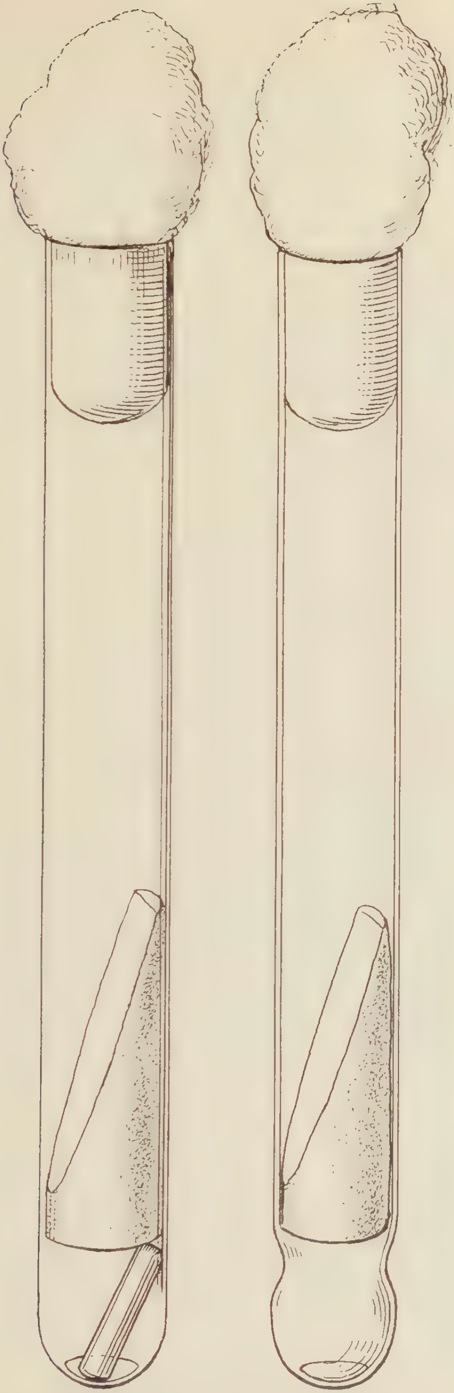

Figure 6. Drawing of Tubes for Glycerinated Potato Medium

Two types of support for holding the pieces of potato out of the few drops of media in the bottom of the tubes are shown. Either the small glass rod or the constriction in the tube works satisfactorily.

autoclaved at 15 pounds for 30 minutes after a little of the bile-fluid has been added to the tubes.

6. *Sabouraud's:* Occasionally it may be necessary to grow molds, yeasts or mycotic microörganisms. This is best done on Sabouraud's medium, which is made as follows:

Dextrose	40.00 gms.
Agar	35.00 gms.
Peptone	10.00 gms.
Water	1000.00 cc.

No titration is necessary. The ingredients are added to the water and the mixture filtered, tubed and autoclaved.

b. *Sealing of tubes:* Because of the slow growth of tubercle bacilli, cultures must remain for several weeks in the incubator. This offers great opportunity for evaporation and even complete dehydration of solid media. To obviate such an occurrence the cotton plugs are sealed in place with paraffin. Tubes properly sealed show no loss of water even after many months of residence in the incubator. Sealing of the tubes is satisfactorily done in the manner related below.

Tubes are placed in an ordinary test tube rack. With scissors all excess of cotton above the mouth of the tube is cut away, the cut end flamed, the plug forced about 1/16 of an inch below the tube mouth, flamed again, and melted paraffin is added to the plug by a dropper while the tube is still warm from the flaming. Paraffin is dropped on the plug of each tube in succession in the rack. This is promptly absorbed. Then, after cotton and tube have cooled somewhat but before they have become cold, more paraffin is added to fill the basin formed by the depression of the plug. This is allowed to cool in a vertical position. The different stages of this procedure are illustrated in figure 7. It has been suggested by Novy and Soule (165) that a warm needle be passed through the plug after it has been sealed in order to allow continuous and sufficient oxygenation of cultures. Such plugs are easily removed with the aid of a sailmaker's awl after the paraffin has been warmed in a bunsen burner flame.

c. *Liquid media:* As has already been indicated, liquid media are very useful for certain purposes, especially the preparation of tuberculin. Many liquid media have been described, but, for the practical purposes which the scope of this book means to embrace, only two need come up for consideration.

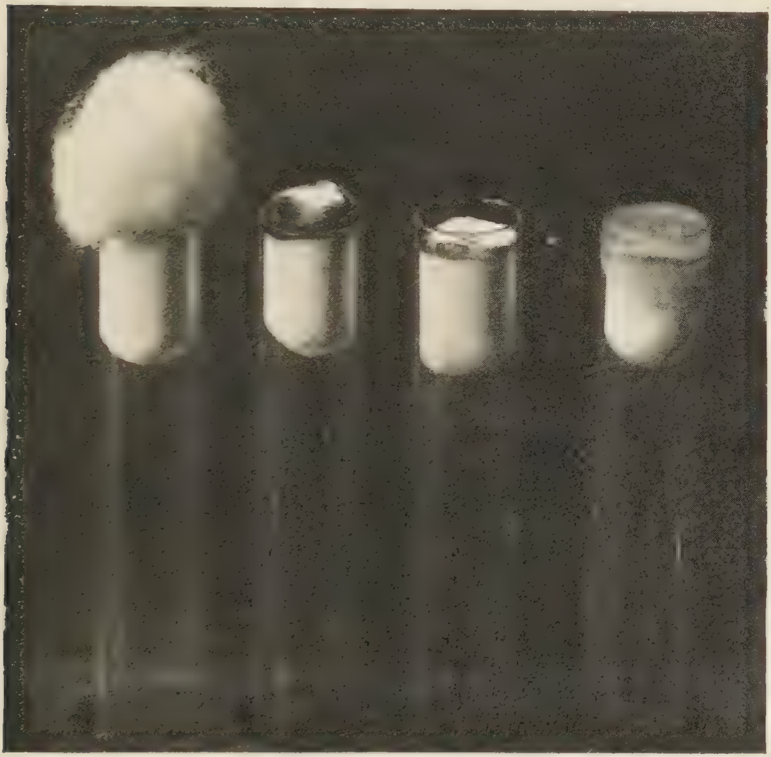

Figure 7. Photograph of Tubes in Various Stages of Sealing

The cotton plugs are shown in the several stages of cutting, depression into the mouth of the tube and sealing with paraffin. Further description is given in the text.

1. *Glycerinated broth:* This broth, a rich yellow, clear fluid, is prepared in much the same way as glycerine agar; the contents being

Lean beef	500 gm.
Peptone	10 gm.
Sodium chloride	5 gm.
Glycerine	50 cc.
Water	1000 cc.

Grind the beef and let it soak in the water in the ice box over night or for 24 hours.
Boil 1/2 to 3/4 hour.
Strain through 4 to 6 layers of gauze.
Slowly add peptone and sodium chloride while broth is still hot.
Filter through paper.
Add water q.s.........................1000 cc.
Titrate (phenolphthalein) to 0.5 per cent acid with N/20 NaOH or
Correct acidity to pH 6.8 (bromthymol blue).
Filter again if necessary.
Add glycerine..........................50 cc.
Bottle.
Autoclave 30 minutes at 15 pounds pressure.

Autoclaving frequently leaves a cloudy precipitate: adequate sterilization can be secured by placing the bottles in an Arnold sterilizer and boiling for one hour.

2. *Potato broth:* This is especially useful for invigorating a languishing glycerine broth culture.

Add grated potato.................. 500 gm. to
Distilled water.....................1000 cc.
Let stand in ice box for 24 hours.
Filter through gauze.
Adjust to about 1% acid.
Bring to a boil.
Filter several times through paper.
Adjust to 0.5% acid with phenolphthalein or
Correct acidity to pH 6.6.
Add glycerine to 4%.
Bottle.
Sterilize as for glycerine broth.

3. *Protein-free broth:* When it is desirable to make tuberculin that is free from proteins derived from media, a synthetic medium such as the following one devised by Long and Seibert (136) may be used, for bacilli grow well upon it:

Asparagine	5.00 gm.
Ammonium citrate	5.00 gm.
Potassium acid phosphate	3.00 gm.
Sodium carbonate (anhydrous)	3.00 gm.
Sodium chloride	2.00 gm.
Magnesium sulphate	1.00 gm.
Ferric ammonium citrate	0.05 gm.
Glycerol	50.00 cc.
Water	1000.00 cc.

3. *Determination of reaction of media:* Most of the organic substances used in the preparation of media have an acid reaction. Tubercle bacilli and indeed most bacteria grow best on media which is near neutral in reaction. It is therefore necessary to "adjust" the reaction by the addition of sodium hydroxide or some other alkali. The required amount of alkali, or acid as the case may be, and the reaction of a given specimen of media are measured by certain methods of determination, the principles of which cannot be gone into at length here, and only a brief statement of principles can be given. An excellent, short, compact statement of these principles is given by Park, Williams and Krumwiede (170).

The true reaction of a solution is determined by its concentration of hydrogen ions or hydroxyl ions.[14] A solution of acid which contains one gram of ionizable hydrogen (H^+) per liter is normal in respect to that acid and is written N/1. Likewise an alkaline solution which "contains an amount of ionizable hydroxyl (OH^-) chemically equivalent to the one gram of hydrogen" is a normal (N/1) alkaline solution. Acid and alkalies used for the purposes with which we are here concerned are in a strength of normal, N/1, tenth normal, N/10, or twentieth normal, N/20 (see below in titration of media). The concentration of hydrogen or hydroxyl ions in a solution is indicated by the symbol pH followed by a numeral, and the pH scale is determined on the following basis.

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[14] In these paragraphs dealing with acids and alkalis, I have drawn from Park, Williams and Krumwiede's (170) summary of this complex field.

Distilled water[15] is considered to be exactly neutral because it contains an equal number of positive hydrogen (H^+) and negative hydroxyl (OH^-) ions. These are continually splitting off from each other in the solution (becoming dissociated or ionized) and are just as readily recombined. In one liter of distilled water there is 1/10,000,000 gram of ionizable hydrogen. Because of the inconvenience of expressing this fraction as 1/10,000,000, or 0.0000001, or 1×10^{-7}, the term is simplified (Sörensen) and expressed as the negative logarithm of the number, or as pH 7. The symbol of pH 7, then, stands for neutrality, because at this pH the number of (H^+) and (OH^-) ions are equal. Solutions containing free hydrogen ions, then, descend the scale in proportion to their increasing content of acid, the stronger the acid the further along the scale from pH 7 toward pH 1. And alkaline solutions, according to the amount of hydroxyl ions, range the scale above pH 7 as is shown in the accompanying chart (table 7) which is taken from Park, Williams and Krumwiede and which shows the pH range of values as compared with "acid and alkali normalities" and hydrogen concentration value per liter of solution.

a. *Indicators* are substances which in the presence of varying amounts of acids or alkalies give different colors to the solutions containing them. By means of various indicators the degree of acidity or of alkalinity may be determined. Two main colorimetric (by use of indicators) methods of "adjusting" the reaction of media are depended upon. These are (1) the titration with phenolphthalein and (2) the comparator method of matching the media specimen containing indicator with a set of standards containing solutions of various pH values and the same indicator.

b. *Titration:* Phenolphthalein is an indicator which is colorless in acid solutions. In alkaline solutions it assumes a pink to red color, depending upon the concentration of alkali.

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[15] Water from some stills, however, is not neutral, as Williams and Sweet (233) have shown, and a sample should be tested occasionally.

It is made up in a strength of 0.5 per cent in 50 per cent alcohol and 10 to 15 drops are used for a determination, which is as follows:

Twentieth normal sodium hydroxide (N/20 NaOH) is poured into a burette. The stopcock is opened and all air forced out so that the NaOH solution fills the burette to the tip. From this point either of two different procedures may be employed.

Table 7

Hydrogen Ion Concentration

	pH values	Acid and Alkali normalities	Ch* values or grams per liter of hydrogen ions
	pH 0.0	N/1	1
	pH 1.0	N/10	1/10
	pH 2.0	N/100	1/100
	pH 3.0	N/1000	1/1000
Acid:	pH 4.0	N/10000	1/10000
	pH 4.5	Color change—methyl orange	3.2/100000
	pH 5.0	N/100000	1/100000
	pH 6.0	N/1000000	1/1000000
	pH 6.8	Color change—litmus	1.6/10000000
Neutral:	pH 7.0	N/10000000	1/10000000
	pH 7.5	Blood	3.2/100000000
	pH 8.0	N/1000000	1/100000000
	pH 8.5	Color change—phenolphthalein	3.2/1000000000
	pH 9.0	N/100000	1/1000000000
Alkaline:	pH 10.0	N/10000	1/10000000000
	pH 11.0	N/1000	1/100000000000
	pH 12.0	N/100	1/1000000000000
	pH 13.0	N/10	1/10000000000000
	pH 14.0	N/1	1/100000000000000

* Ch is the symbol for concentration of hydrogen.

1. *Room temperature method:* Into a casserole 45 cc. of freshly boiled and cooled (not cold) distilled water is placed and to it is added 5 cc. of the medium to be tested and 1 cc. of the phenolphthalein solution. If the solution remains colorless, the medium is acid; if it turns red upon the introduction of the indicator, it is alkaline. As stated above, new media are acid,

and usually titration indicates that an addition of alkali is necessary. The level of the fluid in the burette is read and recorded and this solution added drop by drop to the casserole and the whole stirred with a clean glass rod after each addition of alkali. When the solution in the casserole becomes *faintly* pink and remains so after stirring, sufficient NaOH has been added. Again the fluid level in the burette is determined. The former figure is subtracted from the latter and the amount of N/20 NaOH required to neutralize the 5 cc. of media in the casserole is thus determined. A N/20 solution stands to 5 cc. as a normal solution stands to 100 cc., therefore, the burette-determination indicates the amount of *normal* NaOH which would be required to neutralize 100 cc. of the medium under test. The optimal reaction of medium is 1.5 on the acid side of phenolphthalein-neutrality, if meat infusion is used, or 0.9 if beef extract is the source of nutrient matter.

The following *example* will suffice to illustrate the calculation. It may require 2 cc. of N/20 NaOH to bring the 5 cc. of medium to the neutral point. It would then require 2 cc. of N/1 NaOH to *neutralize* 100 cc. of the medium, or 20 cc. to neutralize a liter. But we titrate the medium to 1.5 on the acid side instead of titrating to the neutral point, and this figure should be subtracted from the 2 cc. This would leave 0.5 cc. of N/20 NaOH to bring the 5 cc. of medium to 1.5 acid, or 0.5 cc. N/1 NaOH to the 100 cc. or 5 cc. to the liter. One should not, however, add the full 5 cc. of normal hydroxide to the liter of medium because of the changes in the buffer solution. It is better to add only one-half the required amount and retitrate.

Acid should not be added to media made too alkaline in the course of titration, as it may produce a markedly unfavorable influence upon bacteria. It is better either to discard media made too alkaline and begin afresh or to add more untitrated media to it (170).

2. *Boiling temperature method:* This method is less reliable than the room temperature method because phenolphthalein is less sensitive at higher temperatures. The water and media are boiled, the phenolphthalein immediately added and titraton accomplished as in the room temperature method.

Titration methods are rapidly becoming superseded by the more accurate method of pH determination. This is true because we are able, by titration, to determine only the total acidity and not the actual ionizable hydrogen or the "actual acidity at a given time."

c. *Comparator method:* Sets of standard tubes with known amounts of acid or of alkali may be prepared. With the addition of given amounts of indicator, the color ranges in the tubes correspond to exact pH values. The indicator with the best color range for standardizing media for growing tubercle bacilli is brom thymol blue (Di bromo sulfon phthalein) with a range of pH from 6.0 to 7.6. The refinements necessary to the preparation of a set of precise standard tubes as recommended by Clark and Lubs (38) requires considerable chemical technique and judgment, but this has been greatly simplified without too great a loss of accuracy by the scheme of Barnett and Chapman (15). In this method tubes containing a given amount of acid and indicator are arranged in pairs with other tubes containing a given amount of alkali and indicator. When light passes through these pairs of tubes, definite colors are given. By varying the amount of indicator in the pairs of tubes a gradating range of colors is produced which by comparison with electrometric standard are found to have fairly precise pH color values. Sets of these standard tubes may be prepared in the laboratory according to any one of several methods given in such standard textbooks as Park, Williams and Krumwiede (170), Zinsser and Russell (241) or Jordan (92). They may also be purchased from a number of chemical or reagent dealers, as La Motte in Baltimore. Such purchased sets are dependable and are not excessively costly. They are to be recommended, for the preparation of tubes in the laboratory is sometimes fraught with difficulties. Brown (25) has recently worked out a system for determining the pH which is both accurate and rapid and at the same time very simple in its execution. It is probably the best method we have. The apparatus is manufactured and sold by the La Motte Chemical Products Co., Baltimore. When standard tubes are used, the colors are best compared in a comparator block. Such a block to be of convenient size should be a cube of about $2\,^1/_2$ inches. It may be made by boring, in the top, two rows of two holes each to a depth of two inches and a little larger than the tubes. Those in each row should be connected by a slit drilled in a horizontal plane through the entire length of the block, for the passage of light.

1. *Determination of the* pH *of media:* In the comparator block, the two holes in the row on the left side are filled with the pair of tubes having the color value of pH 6.8; or, if a single standard tube is used, it is put in the front hole and a tube containing 8 cc. of water and 2 cc. of medium in the back hole of this side. A tube containing 8 cc. of water, 2 cc. of medium and 1 cc. of indicator is placed in the front hole of the other row and a tube containing water in the remaining hole. Then the block is held up toward the window. The color in the two rows should be alike. If the color is too light, as is usually the case, N/20 NaOH is added from a burette until the desired color is obtained. The amount of N/20 NaOH required from the burette is multiplied by 25. This calculation determines the amount of normal NaOH required to bring one liter of the medium to the desired pH value.

The La Motte apparatus mentioned above is provided with a series of buffer solutions reading from pH 4.8 to pH 8.4, four different indicator solutions and a number of small glass cells which may be placed on an opal glass plate with etched pH figures. A few drops of proper buffers in each cell to which is added a drop of indicator very quickly sets up the pH color scale. Determination of the pH of the media is then made by adding a drop of indicator to a cell containing ten drops of medium and comparing the color which the scale just prepared. Gradual addition to the medium of alkali (or acid) may be done and determinations made until a proper color is obtained.

4. *Disposal of cultures:* It should probably go without mention that all cultures which have been discarded because of age, contamination, lack of bacillary growth or for any other reason should be thoroughly sterilized in the autoclave before they are cleaned. Fifteen minutes at 15 pounds pressure is sufficient to render all bacilli entirely innocuous. Tubes are then ready for boiling and cleaning.

Technique of Subculturing Tubercle Bacilli

All transferring should be done over a pan which contains a pledget of cotton soaked with 2 to 5 per cent carbolic acid and a 25 or 50 cc. cylinder filled with the disinfectant. After seeding, the spade is well wiped on the pledget of cotton and then washed by rapid passage back and forth in the fluid in the cylinder. It is then flamed. This is an important precaution because the high content of waxes in tubercle bacilli causes them to sputter and spread in all directions if they are introduced into the flame before the spade has been washed. It is convenient to always

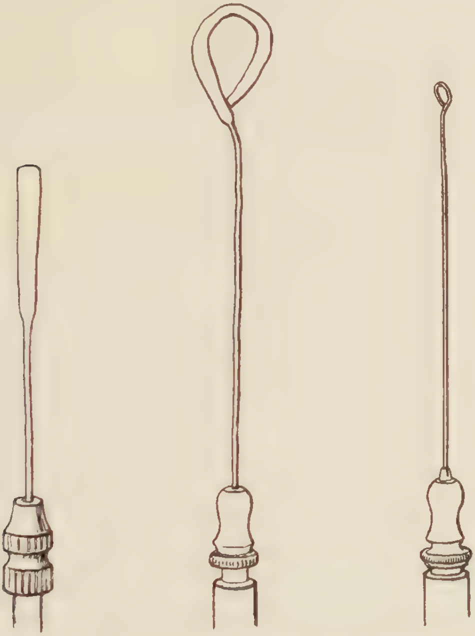

Figure 8. Drawing Illustrating Types of Transfer Spades

The flat spade on the left has been found convenient in the transfer o cultures to solid media; the loop in the centre can be used very satisfactorily for carrying bacilli from broth to broth cultures; and the other represents the commonly used loop found in all bacteriological laboratories. It is especially useful in preparing a drop of suspension for estimating the number of bacilli in the microscopic field.

have the disinfectant in brown or other colored bottles and to use no bottles of that type for other purposes in the laboratory. Types of spades used for transferring are shown in figure 8.

In transferring tubercle bacilli, as in any other work in the laboratory which requires the handling of bacteria, the worker should wear a gown and have his sleeves rolled to his elbows.

1. *On solid media:* Transfer of tubercle bacilli from tube to tube of solid medium is a comparatively simple, easy task. The paraffined plugs should be loosened on all tubes to be used before transplanting is begun. The new tubes should be labeled just before seeding, and only one culture should be on the work-table at a time. It is convenient to place the date of seeding of the parent culture, the name of the strain, the type of medium from which the seed was taken, and the date of subculturing on the label of the tube in such way as that shown in the accompanying schema.

xi/12 GA
Smegma
xii/3

A large load of bacilli spread gently over a large area of the surface of the slant gives the best results. Care should be exercised against digging into the surface. The donor tube is replaced in its tumbler between each transfer as it is not desirable, because of the necessary time required for spreading, to hold donor and recipient tubes in the hand at the same time during the transferring. Donor and recipient tubes are kept in different tumblers. These simple safeguards are of the greatest importance because they will at some time prevent a mistake. They are particularly to be employed in the handling of old, valuable stock strains that have been carried for years and are almost irreplaceable. These considerations apply alike to solid and liquid cultures. Cultures should not be allowed to stand in the light longer than is necessary for transplanting and never in the sunlight. Subculturing should be done every two or three weeks, but bacilli usually grow if several weeks elapse between transfers. They grow much better if the cotton plugs are placed rather loosely and sealing of the tubes is delayed for three or four days after planting. This is an important consideration because they need oxygen and, as Novy and Soule (165) have shown, a small puncture with a needle through the sealed plug facilitates growth.

2. *On liquid media:* In transferring bacilli from broth to broth a large platinum loop is necessary (figure 8). It should measure approximately 8×10 mm. and should be flattened. The culture bottle is placed over a pan on a wire basket or some other form of support. The loop is heated and the mouth and neck of the bottle flamed. The loop is next passed into the bottle, through and under the film especially at a thin edge where active growth is most likely taking place and is then lifted up in a horizontal plane through the growth in such a way that a pellicle of growth the size of the loop is carried away upon it. This is carried into the new bottle of broth which is held at an angle of about 45° over the transfer pan, the loop being brought gently into contact with the fluid, on which the pellicle will float. It is important that the pellicle should float, as no bacilli which settle to the bottom of the fluid will grow. The technique of planting on liquid media is illustrated in figures 9 and 10.

The transfer from liquid to solid media is accomplished easily, but transfer from solid to liquid presents difficulties. The granular, warty growth from solid media all too often sinks to the bottom of the fluid when transfer occurs. Such transfers may be made, however, by placing a small block of solid medium with a thickness corresponding to the depth of the broth in the bottle and seeding that. Bacilli will then grow from the edge of the solid medium to the surface of the fluid. Seeding may also be done on very small (5 mm.) cuts of thin paper which float on the fluid, or on small bits of cork. But what has proved to be a most satisfactory scheme for beginning broth cultures is one originated by Lutz (139) in our laboratory and shown in figure 11. Slants are made in 100 cc. erlenmeyer flasks by pouring in 50 cc. of glycerinated agar. After this has been

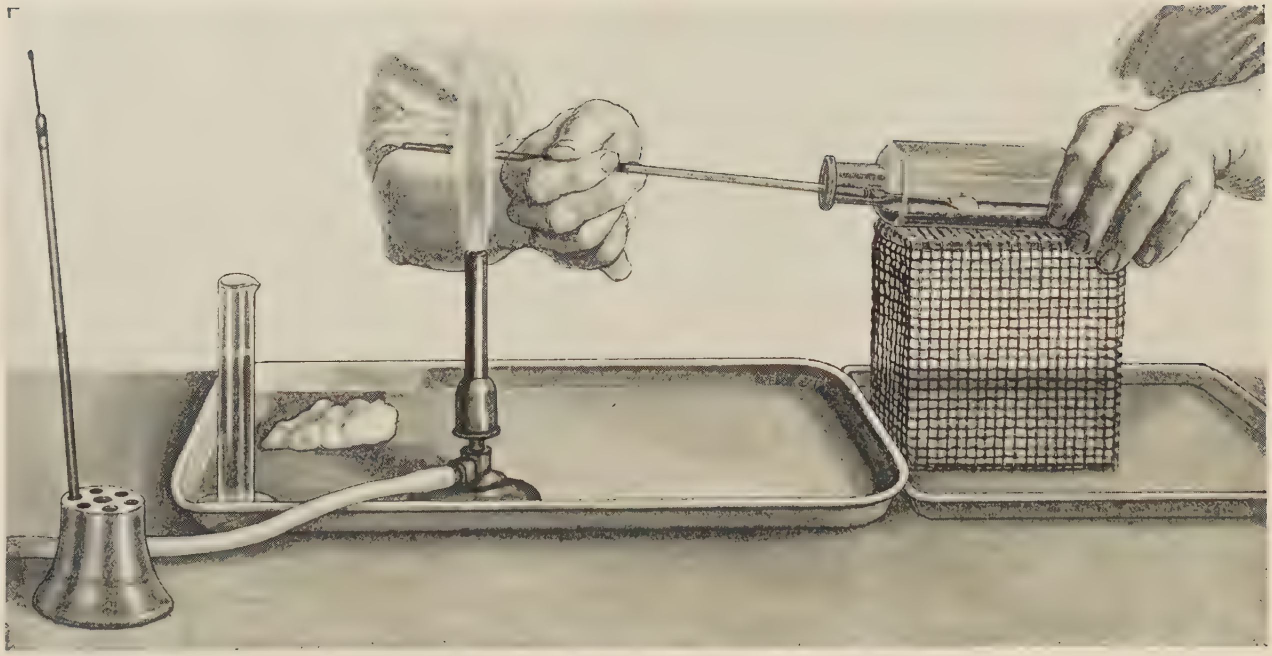

Figure 9. Drawing Illustrating Transfer of Liquid Cultures
The loop has been passed under the growth and lifted up with a disc of the latter lying upon it for removal to recipient bottle shown in figure 10.

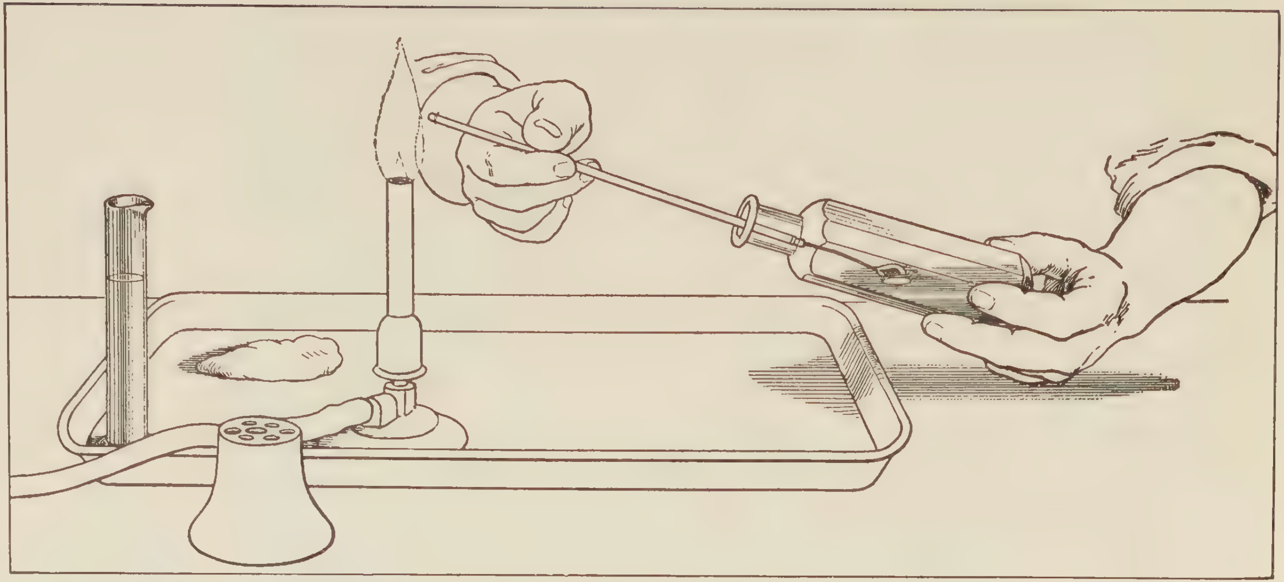

Figure 10. COMPANION DRAWING TO THAT IN PRECEDING FIGURE

It illustrates the second stage in the transfer of liquid cultures and represents the deposit of the film of bacilli upon the surface of the broth. The technique is described in the text.

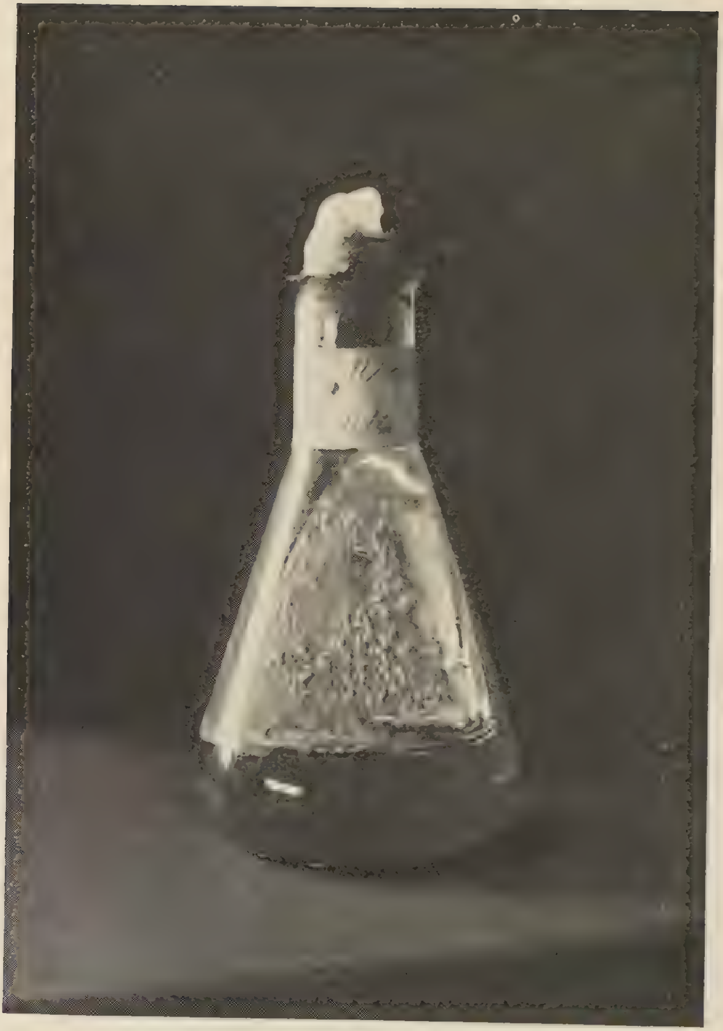

Figure 11. RETOUCHED PHOTOGRAPH OF COMBINED SOLID AND FLUID CULTURE

Bacilli may be seen to be growing from the solid medium, apron-like, over the surface of the broth. Transfer from this liquid to the regular bottles of broth is easily done.

sterilized and hardened in a slant in a plane with the mouth of the flask, 15 cc. of sterile glycerine broth is added and the flask kept in a vertical plane. Then the surface of the solid medium is richly seeded down nearly to the edge of the fluid, the plug is loosely put into place and the flask incubated. Two days later the flask is tightly sealed. Bacilli spread over the surface of the broth by direct growth from the solid medium and so become continuous over the surface of solid and liquid media. Once growth has developed on the broth, its transfer to bottles is easy. If growth does not spread spontaneously over the broth, a pellicle may be dislodged from the agar and allowed to fall to the surface of the fluid where it floats and begins to grow. The method is highly satisfactory.

Inoculation of Media with Body Fluids, Secretions, etc.

Consideration has already been given to the preparation of the various fluids, secretions and excretions of the body up to the point where they are to be actually placed on the medium. Urine, faeces, purulent fluids or those that have not been aseptically removed almost without exception should be run through one of the digestion processes before planting takes place: And this for two reasons. In the first place it removes all contaminating or associated bacteria, and secondly it concentrates the tubercle bacilli from a large bulk of material into the compass of a few drops. Unless, however, the residue from the process is washed (in the case of antiformin) or neutralized (in Petroff's method), growth is much slower in making an appearance and sometimes fails to occur even when bacilli are present. The Griffith method, however, is an exception.

Planting may be done with an ordinary small platinum loop or it may be dropped on the surface of the medium from a sterile pipette. In the latter event, the tubes should be incubated for two days in an inclined plane that will allow the fluid to cover a large part of the surface of the slant. If the tubes are placed in an upright position after planting, the fluid tends to drain off the surface of the slant toward the bottom of the tube. The plugs should be placed in lightly and not sealed for two or three or even four days, depending either upon the amount of fluid introduced with the inoculum or whether the medium begins to dry. Bacilli grow distinctly better if, by this procedure, an abundance of oxygen is admitted to the tube. First plants should always be made upon Dorset's egg, Petroff's or Lubenau's medium. Subsequent plants grow better on Petroff's or glycerinated agar.

Demonstration of Other Bacteria than the Tubercle Bacillus

In sputum in which repeated examination fails to disclose tubercle bacilli, other causative bacteria should be sought. If present, these are usually to be detected by ordinary blood-agar plate methods. The patient is requested to thoroughly rinse his mouth with sterile physiological saline. The specimen is collected in a sterile container, washed in several changes of sterile physiological saline, as outlined in the washing method of Kitasato (p. 61) and, after the final washing, parts of the specimen are placed in a small amount of physiological saline or broth, which is then shaken thoroughly. A particle of the specimen from this solution is streaked several times across a blood-agar plate and the loop carried directly to three or four similar plates in succession for inoculation. These are incubated. It is usually not difficult to isolate a pure culture from one of the plates.

Chapter VII

THE NON-PATHOGENIC ACID-FAST BACTERIA

The discovery of the non-pathogenic acid-fast bacilli excited the greatest possible interest and enthusiasm because, theoretically, they appeared to present relationships between infection in man and the cold blooded animals similar to those existing between small pox and cow pox. They carried immunological implications of great hope. In their study is recorded the story of one of the startling and heroic experiments in all medicine in which Moeller (152) inoculated himself with virulent tubercle bacilli after some preliminary treatments with a strain of non-pathogenic acid-fast bacilli. From 1897 when interest was first keenly awakened in the subject (Moeller (151), Bataillon et al., (16) and others) until 1905, men and women sought these bacteria far and wide, with the result that more than forty different types have been reported.

The term *tubercle bacillus*, it must be emphasized again, should be restricted to the three types of pathogenic acid-fast bacilli—the human, bovine and avian—and should never be applied to other types of bacteria that are acid-fast. The bacteria of this latter group all possess the staining characteristics of the tubercle bacillus and, under the microscope, are morphologically identical with it. They all grow luxuriantly and comparatively rapidly on many different kinds of media and better at room-temperature than in the incubator. Several of them, possibly all, have the capacity to elicit an inflammatory reaction and sometimes the formation of a structure that very closely resembles histological tubercle if it is not identical with it, but the reaction is transient and fleeting and does not progress or produce the death of the animal.

Occurrence in Nature

The story of the discovery of the various non-pathogenic acid-fast bacilli and their application to the problem of immunity to tuberculosis is a fascinating one, and the reader is referred to a series of essays by Krause (109) who has given an excellent though brief account of their discovery and has presented sharply their relation to the tubercle bacillus. My dependence upon these essays has been considerable in the preparation of this chapter.

These bacteria have been found repeatedly in widespread situations in nature but particularly in relation to cold blooded animals. Each newly described bacillus, although bearing close resemblance to others already described, was thought to have a body of characteristics which gave it an identity separate from the others. They have been found repeatedly in many cold-blooded animals—snakes, turtles, fish, especially those commonly found in aquaria, turtles, frogs, blindworms, and many other cold-blooded creatures. While sometimes found in the normal tissues of these animals, they were usually associated with disease or at least with pathological lesion, and, although they are in all probability saprophytic, it is not surprising that the disease in the various cases was attributed to these easily disclosed bacteria. Küster (118), however, felt certain that, at least in the case of the frog, the acid-fast bacillus he worked with was pathogenic, and Aronson (5) has recently isolated a strain of acid-fast bacteria from a fish that is pathogenic for gold fish, frogs, mice, and pigeons, although not for guinea pigs and rabbits.

Other similar bacteria were found to be living an apparently saprophytic existence in close association with domestic animals and man. One strain was discovered to be an inhabitant of the normal urine of horses and cattle and was called the *harn* bacillus; one or two others likewise lived in the intestinal tract of these animals and could be demonstrated in the faeces—the *mist* bacillus; several strains could be cultivated from cow's milk and several others from butter.

Meantime a good number of supposedly different strains were being discovered free in nature: especially on timothy grass and other types of vegetable life.

Thus it becomes evident that these widespread bacteria occur chiefly in three sets of circumstances, namely, (1) widespread in nature, especially in the grasses, (2) in association with cold-blooded animals, and (3) parasitic in warm-blooded animals. There is every reason to suppose that these various types of microörganism are in reality all one or are variants of one germ; that they are all timothy bacilli; that the turtle and the frog and the snake and the fish acquire their acid-fast bacteria from the grasses in the water in which they live and that horses and cattle digest many timothy bacilli with hay and food, some of which turn up in the faeces, urine, milk or other of the excretions of the body. Krause stated that "the unprejudiced and more detached observer could only come to one of two conclusions: either that the matter of non-pathogenic acid-fast bacteria was chaos or that the investigators were studying the same types of microörganisms under different conditions or in different phases which deluded them into believing that the small part of the cycle which was disclosed to them was really the entire life history of the germ."

RELATION TO THE TUBERCLE BACILLUS

1. *Pathogenicity:* When put into animals even in small numbers, the tubercle bacillus initiates pathological change and progressive disease which eventuates in death. When non-pathogenic acid-fast bacilli are put into similar animals, in large numbers, they produce transient pathological change of minor order but the animal does not sicken and die. The bacteria are soon eliminated, apparently not having the capacity to multiply for long in the body, and the animal returns promptly to an apparently normal state. This difference in pathogenicity stands out as the signal difference between these bacteria and the tubercle bacillus. And the fact is of the greatest practical importance when bacteria of uncertain type are inoculated into animals for diagnosis.

2. *Morphology and staining properties:* Although a good deal has been made of differential staining of the nonpathogenic acid-fast bacilli (p. 129), the net impression is that the staining properties are, except for a few rather minor differences, essentially the same for them and tubercle bacilli. There is

likewise nothing in the morphology of the two types to distinguish them.

3. *Serological relationships: "Cross agglutinations," etc.:* The non-pathogenic acid-fasts touch the tubercle bacillus in two other conspicuous ways, aside from their similar staining properties and morphological likeness. They produce cross-sensitization and cross-agglutination reactions with the tubercle bacillus (Koch). An animal may be infected with tubercle bacilli. If tuberculin prepared from timothy, frog, or any other of the numerous non-pathogenic acid-fasts be applied to the skin of that animal, it will elicit a reaction which may not be so vigorous as one produced by a homologous tuberculin but a good reaction, nevertheless. Furthermore, the serum from that animal will agglutinate non-pathogenic acid-fast bacilli. If an animal be infected with any of the nonpathogenic acid-fasts and be tested with tuberculin from a human or bovine tubercle bacillus, it will likewise react, and its serum will produce agglutination of human or bovine bacilli. This "reciprocal sensitization" was re-studied by Krause and Baldwin (115). Moreover, Furth (65) has recently pointed out that the mammalian group can be distinguished from the non-pathogenic by complement fixation. This question deserves further study.

4. *Biological relationships:* If they are so much akin to the tubercle bacillus then, is there not a potential danger of transmutation from non-pathogenic to pathogenic forms? Did all tubercle bacilli once originate from timothy bacilli which by prolonged residence in the intestinal tract or the body of warm-blooded animals gradually become transformed into germs having pathogenicity for cattle, and thus become bovine bacilli? And is it possible that, upon transmission to human beings they became adapted; acquiring traits of pathogenicity for man? It is an engaging hypothesis, but a hypothesis only it is, for, so far, all the evidence is, as convincingly shown by Weber and Taute (229), against the idea of transmutation.

Occurrence in Man

Non-pathogenic acid-fast bacilli may occur in man both in health and in disease, and, although confusion arises only infrequently from their presence, it is important to bear in mind what these possibilities are. The chief secretions in which they may be found are the following: (1) urine, (2) nasal discharge in ozaena, (3) sputum in putrid conditions of the lungs, (4) ear wax or discharge from the ear and (5) the stool. The term "paratubercle bacilli" has been applied to these acid-fasts, exclusive of smegma, that occur in these various relationships to man. The occurrence of these bacilli are as follows: (1) In healthy man the smegma bacillus may be found about the external genitalia, especially in the small collections of smegma under the prepuce and in the labial folds. It also inhabits dirty skin, particularly in areas such as the axilla where the cutaneous folds offer a favorable seat for its development. It has already been pointed out that the smegma bacillus must always be taken into account in the diagnosis of urogenital tuberculosis, and means have been indicated (p. 11) for obviating uncertainty on that score. (2) The nasal secretion (and hence the expectoration) in ozaena frequently contains an acid-fast bacillus which is referred to as a saprophyte, but which may be a source of erroneous diagnosis. Michialoff (147) among others has pointed out the identity of the bacillus of ozaena, which is not acid-fast. (3) In putrefactive conditions of the lungs—gangrene, pulmonary abscess or bronchiectasis—an acid-fast bacillus is found on *rare occasions*. It is saprophytic just as are the diphtheroids found in these and certain inflammatory conditions of the upper respiratory tract. (4) Tuberculous otitis with suppuration is an uncommon disease, and the mere demonstration of an acid-fast bacillus in the pus from the ear does not establish the diagnosis, for as has already been indicated, an acid-fast rod can be frequently found in the cerumen of the ear. Such a germ, to be more than a parasite, must be able to kill guinea pigs. (5) Likewise in the stool of normal people harmless acid-fast bacilli have been described.

The question immediately arises as to how these saprophytic acid-fast bacilli are to be distinguished from tubercle bacilli and the answer has already been anticipated in earlier parts of this chapter. It is this, that only tubercle bacilli set up tuberculous disease in guinea pigs. Recourse to animal-inoculation will always solve the problem of the pathogenicity of a bacillus under examination, and in none of these conditions mentioned above, need any misconception exist very long as to diagnosis. Moreover, it is also generally true that the circumstances under which the saprophytes occur and the safeguards in collecting the specimens to be examined will in most instances give a good clue to the true nature of the microörganism present. Criteria by which differentiation may be determined are given below.

Methods of Differentiation

Fortunately it is relatively easy to distinguish the nonpathogenic acid-fast bacilli from tubercle bacilli. Several methods are given with an evaluation of each.

1. *Animal inoculation:* It has already been stated sufficiently often to make a re-statement seem redundant that the nonpathogens never produce disease and death of animals (guinea pigs). Tubercle bacilli invariably do so. No test of differentiation could be more certain or could have less equivocation.

2. *Cultivation on media:* In general there are well-marked differences between these bacilli and tubercle bacilli. The former grow a good deal more rapidly, covering the surface of the slant with a thin, spreading growth in four or five days, which, in the case of timothy, lepra and several others, assumes an orange-colored pigmentation at a week or ten days. The fact that they grow at room temperature has been used as a differential method. If growth occurs under these circumstances when urine or other secretions are cultured and left at room temperature, the growth is supposed to be non-pathogenic bacilli, for the tubercle bacillus appears not to grow at so low a temperature. The tubercle bacillus, however, will occasionally resemble the non-pathogens in its properties for growth and, more commonly, the non-pathogens will grow slowly, much like the tubercle bacillus, so that while cultural characteristics will usually furnish a good lead, this mode of differentiation

has proved to be less sharp and decisive in practice than on paper. And of still more importance, at least theoretically, is the fact that the tubercle bacillus *may* be present along with the smegma or other acid-fast germ and would fail to grow under such circumstances. Yet this would not prove its absence.

3. *Differential staining:* Numerous staining methods to distinguish between these two groups of bacteria have been devised, most of them centering about both the alcohol and the acid-fastness of bacilli. A good many claims for these methods have been made, but it is probably true that none of them is absolute or dependable in its differentiation. Grethe (73), in 1896, stained with carbolfuchsin, washed with water and, without acid, decolorized and counterstained with a saturated *alcoholic* solution of methylene blue. He claimed that all smegma bacilli were stained blue while true tubercle bacilli retained the red dye. But others were unable to find that *all* smegma in a preparation were decolorized by this method.

The method of Bunge and Trantenroth (30), with certain exceptional qualifications, has received some confirmation. It and a few other methods will be given.

a. *Chromic acid (Bunge and Trantenroth):*

Allow preparations on slides to dry without fixation by heat.		
Place in absolute alcohol	3	hours.
Let stand in 5% chromic acid	15	minutes.
Stain in the usual way with carbolfuchsin	3	"
Decolorize with dilute sulphuric acid	2–3	"
Counterstain with a concentrated alcoholic solution of methylene blue	5	"

By this method smegma bacilli take the blue stain and tubercle bacilli retain the fuchsin. Dahms (45), testing this method, stained 173 specimens of human tubercle bacilli (22 from sputum and 151 from culture) and found them always red. He stained 63 specimens of smegma and found them always blue. But the authors of the method stated that *occasionally* (unless 100% alcohol were used) all smegma would not be completely decolorized.

b. *Corallin-alcohol* (*Pappenheim* (168)):

Heat preparation as usual with carbolfuchsin for a short time, up to boiling point.
Pour off excess stain.
Without washing, decolorize and counterstain by adding and slowly pouring off (3to 5 times) the following staining solution:

Absolute alcohol	100 parts.
Corrallin	1 part.
Methylene blue to saturation	
Glycerine	20 parts.

Wash quickly in water.
Dry.
Examine.

The tubercle bacilli remain red and the smegma are supposed to be destained and then to take the blue.

c. *Absolute alcohol* (*Honsell* (88)):

Smear and stain with carbolfuchsin (steam) 3 minutes.
Destain with acid alcohol (absolute alcohol 97 cc. and HCl 3 cc.) .... 10 minutes.
Counterstain with alcoholic methylene blue (½ dilution).

Some (but not all) smegma will be decolorized.

4. *Antiformin:* It has been claimed (208) that 15 per cent anti-formin completely dissolves smegma and numerous other nonpathogenic acid-fast bacilli in a half to one hour while human and bovine bacilli remain intact and are morphologically recognizable even after several days' stay in 50 per cent antiformin.

Résumé: Weber (228) has made a careful study of the various differential staining methods proposed and decided that the two types of bacteria could usually be distinguished from each other by careful differential staining, especially if Honsell's method were used. But in a matter of so much importance where staining or other differential methods are of question-able completeness, the only certain method of differentiation between pathogenic and non-pathogenic acid-fast bacilli would appear to be animal inoculation. Wherever any doubt at all exists as to the nature of a bacillus in question, animal inoculation should certainly be made. Removal of a normal kidney has been performed (Walker (223)) on a basis of finding smegma bacilli in the urine. It is possible by meeting certain very exact conditions to be sure by smear and stain as to the identity of the microörganism. But I cannot too strongly emphasize the great importance of reliance upon animal inoculation as the certain method of differentiation where these conditions cannot be fulfilled. Guinea pigs succumb invariably to human or bovine bacilli and never to the non-pathogens.

PART III

DIAGNOSIS BY USE OF TUBERCULIN

CHAPTER VIII

TUBERCULIN AND ITS PREPARATION

It does not fall within the compass of this work to discuss in any detail the nature of tuberculin or the underlying principles upon which it acts, even were the latter more perfectly understood. A very lucid, brief and compact account of tuberculin has been recently given by Krause (108). Hamman and Wolman (80) have made an admirable clinical study of the diagnostic and therapeutic use of tuberculin, and their book should be known to all who administer the product to patients. Only such facts and figures will be given here as are deemed necessary in the presentation of the aspects of the question to which we are limited. In preparing this and the following chapter I have frequently had recourse to the work of Hamman and Wolman.

Tuberculin, *per se*, is a non-toxic product. Large amounts of it may be injected with impunity into animals that are free from tuberculous infection, the animals remaining in perfect health and showing no appreciable signs whatever of intoxication. Nor do they subsequently develop symptoms of illness from such injection. But when it is introduced even in small quantities into animals or men with tuberculous infection, it may produce a profound constitutional reaction and even death. Likewise the product is without the capacity to elicit reaction or inflammation in the skin of normal animals; but in the skin of animals or man infected with the tubercle bacillus its introduction is followed by characteristic and specific inflammation. A discussion of the underlying processes of this matter would carry us too far outside the scope of this work, and the reader is referred to the two works mentioned above.

Tuberculin is an inclusive term. It is used generically, as

Hamman and Wolman point out, to designate any solution containing either dead tubercle bacilli, intact or ground up, or any soluble or insoluble products of the tubercle bacillus. It may and usually does include the ingredients of the broth on which the bacilli were grown, although it may be prepared by growing bacilli on protein-free media or may be made from bacilli washed free of media. Many different tuberculins have been prepared and used clinically, each one alleged by its proponent to possess superior qualities. Hamman and Wolman described more than fifty kinds of tuberculin and did not complete the list. A few of the important tuberculins will be very briefly described.

PREPARATIONS MOST COMMONLY IN USE

1. *Old Tuberculin* (*O.T.*)*:* In the manufacture of OLD TUBERCULIN, tubercle bacilli are grown four to six weeks on glycerinated broth. Broth and bacilli are then boiled for one hour, filtered through a Berkefeld filter, the filtrate evaporated at 50 to 55°C. over a water bath to one-tenth of the original volume, filtered through paper in an Arnold sterilizer, sterilized in the Arnold three successive days at 52°C. for an hour each day, placed in bottles, a few cubic centimeters injected into guinea pigs as test for viable bacilli and the remainder stored in the ice chest. The product is, therefore, highly complex: it contains the soluble ingredients of the broth as well as whatever soluble bacillary material may have been formed in the broth during the life of the bacilli and whatever may have been separated from the bacilli by boiling. It is a highly concentrated substance and is irritating to the tissues unless used in dilution. It is a clear, amber or brown, oily liquid containing glycerine in 50 per cent strength, since the medium before evaporation contained 5 per cent of this ingredient. This is Old Tuberculin as it was originally prepared by Koch (99).

A good many clinicians, especially those working with children, deem it wise to use a control solution that contains the media ingredients present in old tuberculin. Such a preparation, known as "O.T. control," may be prepared by subjecting sterile glycerinated broth to the same conditions of boiling, filtration and evaporation as employed in the preparation of Old Tuberculin itself.

2. *Bouillon Filtrate* (*B.F.*): This preparation was first made by Denys (48) in 1905, in an effort to obtain a solution of bacillary products unaltered by heat, chemicals or mechanical procedure. It is prepared by passing six-weeks-old glycerinated broth cultures of tubercle bacilli as for O.T., unheated, through a Berkefeld filter. The filtrate is collected aseptically and is called Bouillon Filtrate or B. F., as its name implies. As with all preparations of tuberculin, this should be tested for viable bacilli by injection into animals. The only specific elements present in this product are those soluble substances formed in the media during the life of the cultures. These substances are in all probability derived from the metabolism of the bacilli and a certain amount of bacillary disintegration.

3. *Bacillary Emulsion* (*B.E.*): Koch made this product with the idea of treating patients with dead bacilli influenced as little as possible by manipulation. The preparation consists of bacilli that have grown on glycerinated broth, have been separated by filtration from the latter but not washed. They have then been dried and ground and taken up in a 0.5 per cent suspension in a solution made of equal parts of distilled water and glycerine. Each cubic centimeter contains 5 mgm. of pulverized bacilli.

4. *Water Extract* (*W.E.*): This preparation is used largely for experimental purposes. It is free from products of the media, and in its preparation an effort is made to obtain a solution of pure tuberculo-protein. Bacilli are washed free of media (until the filtrate no longer gives a protein or chloride reaction), dried in vacuo, pulverized in a mill and extracted with water. Precise directions for making this product are given on p. 262.

We have in these few tuberculins essentially all that is offered in principle in the long list which have been promulgated. Old Tuberculin by virtue of its concentration probably should be used for diagnostic purposes. Bouillon Filtrate, on the other hand, is a better therapeutic agent because of its relative weakness. Bacillary Emulsion is also used therapeutically and Water Extract has found a significant place in experimental work.

Besides filtration through the Berkefeld, an additional guarantee of the freedom of the preparation from viable tubercle bacilli should be secured by injecting subcutaneously from 2 to 5 cc. of each new lot of tuberclin prepared into each of two normal guinea pigs. The latter should be examined at autopsy six to eight weeks later, and the preparation should not be applied to patients until after this examination.

It is customary to put a preservative in stock tuberculin, but this is unnessary if strict asepsis is employed. Indeed, a valid objection may be raised to the use of preservatives because, unless added with the greatest care, they produce a certain amount of precipitation. There is no question, however, that the addition of phenol in 0.25 per cent or of 0.33 per cent tricresol gives an added protection against occasional contamination. It is desirable to place a layer of toluol on the water extract.

Caution: Tuberculin is a potent substance when brought into contact with a tuberculous individual. It should always be prepared under a hood, especially when there is occasion to handle pulverized bacilli. In handling tuberculin, as in making dilutions or in administering it to patients, one should be very careful never to spray any of it about the room or in any way to allow it to be evaporated rapidly, as over a flame. Serious reactions may follow.

Standardization of Tuberculin

Although it is obviously important and desirable to have a reliable means of standardizing tuberculin, no scheme has yet been devised that has had wide or uniform acceptance. Long (135) has recently given a good criticism of existing methods.

As indicated below, there are three general types of test for standardization.

1. *Lethal dose method:* Numerous methods have been based upon the lethal dose of tuberculin for tuberculous guinea pigs,

and an unknown tuberculin is standardized in this way by being compared with a standard tuberculin. A number (12 or more) of equal sized guinea pigs are infected with a uniform dose of tubercle bacilli and after the lapse of three or four weeks are divided into two lots. The animals in one lot are subjected to the injection of increasing amounts of a standard tuberculin, from 0.5 to 3 cc., each animal receiving one injection. Those in the other lot receive similar doses of the unknown tuberculin. The minimal lethal dose in 24 hours is determined in this way for each tuberculin and, in accordance with whether the unknown product is more potent or less than the standard, it is either properly diluted to correspond to the standard or is rated as substandard. A dose of 0.5 cc. of a standard tuberculin should be lethal for tuberculous guinea pigs in 24 hours. There are several variations of this method, which is originally Koch's (100) own. The important ones are that of Schroeder and Brett (193) and that of Doenitz (49).

a. *Evaluation:* This method is prodigal of guinea pigs, is gross and gives no unit for measuring doses. It cannot take into account the amount of tuberculosis in the guinea pigs on which the test is made. And this is perhaps as important a factor as dosage of tuberculin when the issue is to be settled by death of the animal (135).

2. *Intracutaneous method:* Other methods have rested, not on the lethal dose of a given tuberculin, but on the capacity of the product to elicit intracutaneous reactions when injected into infected guinea pigs. Roemer (186), Lewis and Aronson (128), and others have devised schemes based on the hypersensitive reaction of the skin. Details of the latters' test are as follows:

Guinea pigs of 200 to 300 grams in weight, infected intraperitoneally each with 0.1 mgm. of tubercle bacilli should be, 18 or 20 days later, ready for the test which consists of injecting intracutaneously into shaved cutaneous areas adjoining each other increasing dilutions (in physiological saline) of the unknown tuberculin ranging from 0.02 mgm., through 0.01, 0.005, 0.002 down to 0.001 mgm. The five injections are given similarly to each of six guinea pigs. Likewise into six other guinea pigs from the same infected lot, similar dilutions of a known "standard" tuberculin are injected. All dilutions are brought to a constant bulk of 0.1 cc., the preferable size of the dose, by the addition of the diluent. A tuberculin syringe and a short, finely pointed platinum needle of 26 gauge is recommended. The readings at 48 hours are taken as maximal, and the reactions induced by the unknown tuberculin are compared dose for dose with those of the standard product.

Later, Aronson (4) suggested that into each animal in the lot there be injected the same sized dose of the standard and the unknown tuberculin. He stated that any tuberculin should be considered to be of "standard" potency if a dose containing 0.001 cc. of the original product produces distinct redness at the site of intracutaneous injection.

a. *Evaluation:* This method has several things to commend it. Its one drawback is the fact that not all guinea pigs under given circumstances of infection react constantly, for there is some individual variation among them. In a measure this constitutes an inherent objection to the method, although the use of a larger number of animals should generally meet the objection satisfactorily.

Complement fixation and precipitation reactions have also been recommended but they have in general been found to be not constant and possibly not specific in reaction.

3. *Testicular or spermatocytic method:* All of the tests mentioned are based on the hypersensitive or allergic response of the infected animal to tuberculin. Long (135) has reported that the testicle (especially the spermatocyte) of the infected guinea pig is extremely hypersensitive to tuberculin. He has based a test for standardizing tuberculin upon this fact. It would appear that the spermatocytic reaction will "detect about one-tenth of the minimum quantity detectable by the skin test," and that at the same time it is much more constant than is the latter. The preliminary part of the test consists of subcutaneous infection of male guinea pigs of about 400 gm. with a strain of tubercle bacillus of low virulence (R1 Saranac Laboratory see page 232). After the lapse of one month the animals are sensitive and the test may be performed. Dilutions

are so made that the amount of tuberculin in the various doses will be 0.1, 0.01, 0.001 and 0.0001 cc. of the original, and the total volume to be injected is always 0.1 cc. With solid preparations such as B.E. or tuberculo-protein the suspensions should be so made that actual amounts injected are 0.01, 0.001, 0.0001, and 0.00001 mgm. The injections are made into the middle of one of the testicles through the scrotal skin after it has been washed with alcohol. The testes must be gently forced into the scrotal sac and held there until the injection is made. The injection does not appear to be painful. Two animals are used for each dilution, and the strongest dilution is injected into two normal, uninfected controls.

The testicles are examined histologically. The animals may be killed 36 hours after the injection or two to four weeks afterward. In either event sections are made and examined with the microscope. The histological changes present after the longer period are much more clearly recognizable. For a description of the changes in the tissue at the two periods, reference should be made to the original.

Long suggested that the quantity of tuberculin just sufficient to destroy spermatogenesis in a majority of the tubules constitutes the spermatocytic unit.

a. *Evaluation:* Long pointed to the delicacy, constancy and absolute specificity of the reaction and to the convenience and ease of permanent record that the test offers. He also recognized the disadvantage of the necessary length of time and of technical labor involved. Whether the delicate reaction is so sensitive as to respond to very weak tuberculins cannot be said since the test has not yet had sufficiently wide application. It has a good deal to commend it.

After a just appraisal of these various methods, it must be obvious that, valuable as one or another of them may be, the most important consideration to be borne in mind in the making of tuberculin is in the standardized method of its preparation. As Baldwin, Petroff and Gardner (14) have pertinently remarked, uniformity and a strict adherence to a standard technique both in the matters of media, strain of bacilli, age of growth, etc., as well as in the actual preparation of each lot of tuberculin, constitutes the best guarantee of a standard product.

Dilution of Tuberculin

All tuberculins are given an arbitrary titer of 1000 milligrams per cubic centimeter. But the kind of tuberculin should be mentioned with the dilution, because, for instance, one milligram of Old Tuberculin is much stronger than the same quantity of Bouillon Filtrate.

The diluent of choice is a filtered c. p. physiological saline (0.85 per cent) solution, although it is usually recommended that a preservative be added such as phenol in 0.25 per cent strength (called "carbol-salt" solution) or tricresol in 0.25 to 0.33 per cent strength (p.138). Dilutions, especially high in the scale, should be made every ten days or two weeks if diluted with "carbol-salt" solution, or every three weeks if tricresol is used, for even the small amount of preservative may cause slight precipitation of the tuberculin. Dilutions with physiological saline alone, however, may be preserved a longer time. At the Saranac Laboratory the use of three pipettes is recommended for the dilutions.

No. 1. A one-tenth cc. pipette graduated in hundredths for first dilution.
No. 2. A one-cc. pipette graduated in tenths for the next higher dilutions.
No. 3. A ten-cc. pipette graduated in tenths for the highest dilutions.

These may be sterilized between each use or they may be kept immersed in a jar of 2 per cent carbolic acid and rinsed in the diluent each time before use.

With the diluent and pipettes provided, dilutions may be made in the following way:

1 cc. tuberculin = 1000 mgm.

Dilution A 0.1 cc. tuberculin + 0.9 cc. diluent
0.1 cc. = 10.0 mgm.

Dilution B 0.1 cc. of Dilution A+0.9 cc. diluent
0.1 cc. = 1.0 mgm.

Dilution C 0.1 cc. of Dilution B+0.9 cc. diluent
0.1 cc. = 0.1 mgm.

Dilution D 0.1 cc. of Dilution C+0.9 cc. diluent
0.1 cc. = 0.01 mgm.

Dilution E 0.1 cc. of Dilution D+0.9 cc. diluent
0.1 cc. = 0.001 mgm.

Dilutions may be carried out in this manner to any desired strength. Usually for intracutaneous testing it is sufficient to stop with Dilution C, 0.1 cc. of which contains 0.1 mgm. of tuberculin, as this is the usual dose. For therapy, on the other hand, especially in the beginning, dilutions are carried out to where 0.1 cc. contains 0.000,01 mgm. Where such high dilutions are required, the steps may be abbreviated by using a larger amount of diluent, as follows:

Dilution A 0.1 cc. tuberculin+9.9 cc. diluent
0.1 cc. = 1.0 mgm.
Dilution B 0.1 cc. Dilution A+9.9 cc. diluent
0.1 cc. = 0.01 mgm.
Dilution C 0.1 cc. Dilution B+9.9 cc. diluent
0.1 cc. = 0.000,1 mgm.

and so on.

Chapter IX

DIAGNOSTIC APPLICATION OF TUBERCULIN

The Tuberculin Reaction

Opinion as to the helpfulness of tuberculin in the diagnosis and treatment of tuberculosis has varied a great deal. For a number of years it was esteemed a valuable diagnostic and therapeutic adjuvant, but it was later discarded by all but a few who understood better the nature of its action and knew the limitations of its worth. Even at present its status as a diagnostic agent is, in general, moot, with the result that in adults it is only occasionally employed. The difficulty in evaluating the helpfulness of tuberculin in a diagnostic study is due to the fact that tuberculin causes a reaction in those infected with the bacillus of tuberculosis but it does not enable us to distinguish between infection and clinical disease. The reaction is specific but is not selective among those that react. As will be pointed out later, there are definite indications for the use of the substance in diagnosis. But it should be remembered that a reaction to tuberculin indicates only that the reactor has tuberculous infection and that there may be a vast difference between tuberculous infection and the clinical disease, tuberculosis.

The patient in whom tuberculosis is suspected but cannot be proved is precisely the one in which a dependable diagnostic test would be of greatest worth, but, in such a case, a reaction to tuberculin (e.g., subcutaneous test) can usually do no more than add another link to the chain. The tuberculin reaction of and by itself cannot make the diagnosis, because approximately one-half of healthy adults will react to the (subcutaneous) tuberculin test. If it cannot be used, then, in circumstances such as this where a reliable test is most needed, may it not as well go into the discard? Why should we persist in performing a test that is likely to carry no essential significance after it has been performed? The answer is that we expect too much from the test.

In evaluating results of the diagnostic application of tuberculin, the type of test has a good deal to do, for there may arise several circumstances in which a reaction of a certain type may do a good deal in tipping the scale for or against a diagnosis of tuberculosis, especially in children. These will be considered later when an appraisal of the several different types of test is attempted.

1. *Basis of reaction:* It is now recognized by everyone that people or animals in the normal state do not react to the injection of tuberculin but that, once infected with the tubercle bacillus, they do react, sometimes violently, and this irrespective of whether the reacting body possesses merely anatomic tubercle or whether it is diseased with clinical tuberculosis. Injection into animals of killed tubercle bacilli (see p. 262) or of the non-pathogenic acid-fast bacilli will produce a transitory sensitiveness to tuberculin, but such a phenomenon does not concern us in this connection.

There are important differences between the serological phenomena of hypersensitiveness and immunity, such as agglutination, precipitation, etc., and the hypersensitiveness underlying the tuberculin reaction. These matters have been briefly commented upon in chapters VII and XI and will not be repeated here.

2. *Types of reaction:* Three different types of reaction—the *local*, the *focal* and the *general*—may occur to the injection of tuberculin in the infected body. The *local* reaction consists only of an inflammation at the site of application of tuberculin, which generally begins to develop at about 12 hours after the application, reaches its height at 24 hours or later and has nearly always subsided by the third or fourth day. It is characterized by redness, swelling, and induration of the skin and, rarely, by necrosis and ulceration. The diameter of the area is seldom greater than one centimeter, when applied clinically in patients (Pinquet test). But in animals, where much

larger doses are required, a reacting area should measure as much as one centimeter in one diameter before it is regarded as being positive. In animals, necrosis at the center of the reacting area is common.

For a long time it has been well known that tuberculous tissue itself is extraordinarily sensitive to tuberculin and that it practically always becomes inflamed if sufficient tuberculin is put in the body. This inflammation about the focus of tuberculosis constitutes the *focal reaction* and is truly specific When tuberculin is introduced subcutaneously and the *focal* reaction develops, there occurs simultaneously a *constitutional* reaction, characterized by fever, tachycardia, malaise, and exacerbation of focal symptoms. The reaction continues for 24 or 48 hours or a little longer and then disappears rather promptly. A most rational explanation of this reaction has been given by Krause (113) who believed that it comes about as a result of absorption of products from the focus of disease—most likely products from the degraded or broken down animal-tissue within the focus.

3. *Failure of the infected to react:* The tuberculin reaction is specific. It occurs only in the body of the animal or man that is infected with tubercle bacilli and is called forth only by the introduction into the infected body of tubercle bacilli, living or dead, or products of these microörganisms. Furthermore, the reaction never occurs when tubercle bacilli or their products are put into normal animals.

In addition to the fact that no normal, non-infected animal or man will react to tuberculin, there are several conditions in which the body fails to react to tuberculin even when it is infected or diseased. A negative reaction, when these conditions can be ruled out, may be of great importance, since it rules out tuberculous infection. Occasionally *undernourished children* that have been infected as a result of intimate exposure to tuberculosis in the home fail to react when first tested. A large percentage of these develop the power to react, however, after a few months of change of diet and regimen. Frequently, a patient who is ill with *far advanced tuberculosis* fails to react to tuberculin, but far advanced tuberculosis is ordinarily so obvious that one would scarcely be led into error by a negative response under such circumstances. During the height of or just after several of the *intercurrent infections*, notably measles and influenza, and in *high, continued fever*, there is a diminution in the strength of the reaction or a disappearance of it altogether. *Pregnancy* is said to frequently exert the same influence upon the reacting capacity. Persons who have had tuberculosis or tuberculous infection that has *healed* or that is so thoroughly well encapsulated that little or no interchange occurs between the interior of the lesion and the circulation will also fail to give a reaction to tuberculin. Patients who have been *under tuberculin treatment* for a long time frequently fail to react, probably because, as a result of the repeated, low grade inflammatory, focal reaction and subsequent fibrosis resulting from the application of the tuberculin, the foci of tuberculosis have gradually become more and more shut off from the circulation, that is, have undergone healing. The reaction is also absent during the *incubation period* of the infection, which, in animals, usually lasts from 10 days to two or even three weeks after the infection, the time depending upon the amount of the infecting dose.

4. *Secondary reactions:* When infected man or animal is given a subcutaneous test two to five or six weeks after a cutaneous or conjunctival test has been done, there is now and again a "lighting up" of the area of the former test, even if there were no reaction at the site of the first test. It has been reported that at times such reactions are so marked as to cause a discontinuation of the administration of tuberculin for either diagnosis or treatment, but as a rule these reactions are entirely negligible and of no importance.

We have now, after this brief mention of the tuberculin reaction and its irregularities, to consider several of the more important tuberculin tests, the methods employed in performing them, and an interpretation of them.

CUTANEOUS TESTS

1. *The cutaneous test of Pirquet:* Tuberculin will produce a reaction in the skin of infected people if it is applied in any

one of several ways although it should be borne in mind that the sensitiveness of the skin varies a good deal in different parts of the body, the skin of the back being least sensitive. Recently it has been reported that in symmetrical situations of the body sensitiveness may vary. The flexor surface of the arm is sufficiently sensitive for satisfactory testing, and it is here that most of the tests are performed.

a. *Mode of application:* The Pirquet test is a very simple and at the same time one of the most useful tests we have. The skin over an area four or five inches in length on the flexor surface of the forearm is washed with 70 per cent alcohol and allowed to dry. Near the proximal border of the sterilized zone a drop of undiluted Old Tuberculin is placed and about four centimeters distal to this a drop of O.T. control (see p. 136). Then with a scalpel that has a particularly sharp tip three linear scarifications parallel to the long axis of the arm are made into the skin; the first about four centimeters distal to the drop of O.T. control, the second through this drop of fluid, and the third through the drop of Old Tuberculin. The scarification should be about 5 mm. long and of a depth just sufficient for the appearance of one or two minute points of blood, but not deep enough to cause manifest bleeding (Hamman-Wolman). Stretching the skin during the scarification is helpful. The first scarification constitutes a control to trauma and the second to any protein or other ingredient in the media to which the individual may be sensitive. The drops of solution should remain for ten minutes after scarification and then each should be dried off with a separate sterile sponge, care being taken that none of the tuberculin is carried into either of the two distal incisions. No dressing is necessary. Pirquet devised a borer for making the abrasion but the use of such an instrument causes pain and a certain amount of traumatic reaction.

b. *Interpretation and appraisal:* The reaction is observed after 24 hours and, where it is convenient, again after 48 hours. Its individual variations as to extent are considerable, reactions ranging anywhere from a very slight redness of 2 or 3 mm. with a little induration to an inflammatory process involving the larger portion of the forearm, but fortunately, this latter is exceptional. There is less variation in the time relations of the reaction, for slight redness and induration are usually detectable at eight or ten hours after making the test and the reaction has usually faded a good deal at 48 hours.

It is preferable to measure the degree of induration and inflammation with a caliper, but they may be interpreted relatively according to some such scale as the following one devised by Hamman and Wolman:

"1. *Negative Reaction:* No appreciable difference between the tuberculin areas and the control.

2. *Slight Reaction:* Definite but slight redness with some infiltration.

3. + *Reaction:* A wider area of redness with a definitely raised center.

4. + + *Reaction:* Wider area of redness with more marked infiltration than +.

5. + + + *Reaction:* Unusual redness and wide area of infiltration. All cases which go on to vesiculation."

This test is relatively rough (see p. 151). At least it is not as delicate as the intracutaneous test to be described later, for, when applied to large numbers of people of different ages, the latter test has an incidence that is at least 10 per cent greater than the cutaneous test. But it is interesting and important to recall that the two tests are nearly parallel in the first two or three years of life. A negative Pirquet test should be promptly followed by an intracutaneous test if there are reasons to suspect the presence of infection.

The Pirquet test is elicited more readily when it is repeated, provided the second test be performed within a month or two of the first. If the second be done at or near the site of the first, the second is a more extensive reaction. This is true, however, of most tuberculin tests.

Reference has already been made to the several circumstances in which failure to react is likely to occur in the presence of tuberculous infection. These easily recognized conditions ruled out, the failure to react indicates freedom from tuberculosis. And this is the great value of the negative test whether in children or adults. Just as a reaction may add sufficient information to that already in hand for a provisional diagnosis of tuberculosis, failure to react may, when the circumstances are similar, justify either a diagnosis of non-tuberculous disease or indicate that a period of observation is necessary before diagnosis can be made.

2. *Intracutaneous test of Mantoux* (*145*): It has already been intimated that this test is more delicate than the cutaneous test. It has the advantage of accurate measurement and concentration of dosage. It yields a somewhat higher incidence of reactions than the Pirquet test does and is the test of choice if one is seeking epidemiological data.

a. *Indications:* It is indicated where symptoms or signs or history point to tuberculosis and the cutaneous test fails to give a reaction. In a good many clinics it is routine.

b. *Dosage:* Old Tuberculin should be used in this test in such dilution that 0.1 cc. of the solution contains 0.1 mgm. of the substance. Dilutions may be made as follows: (see also preceding chapter.)

Dilution A	0.1 cc. O.T. + 9.9 cc. diluent 0.1 cc. equals 1.0 mgm.
Dilution B	0.1 cc. Dilution A + 0.9 cc. diluent 0.1 cc. equals 0.1 mgm.

Accurate measurement of so small a quantity of tuberculin should be made with a one-tenth cc. pipette graduated in hundredths of cubic centimeters. The diluent should be kept in stock and may be sterile physiological saline made from distilled water with or without either phenol in 0.25 per cent strength or tricresol in 0.33 per cent strength as a preservative. Either of these preservatives will cause a certain amount of precipitation so that the dilutions, particularly the weaker ones, become impotent in a few weeks (p. 142). Where strict cleanliness and asepsis are employed, there is no need to use a preservative in the saline diluent; in this case the potency of the tuberculin persists for a longer time.

c. *Mode of application:* A 1 cc. tuberculin syringe (Becton-Dickinson) fitted with a 25 or 26 gauge, one-half inch needle is preferable. The flexor surface of the forearm is cleansed with alcohol as for the Pirquet test. The skin is held slightly taut by squeezing upon the extensor surface of the arm, and the very tip of the needle is forced by a slight, twisting motion into the superficial layers in a plane almost parallel to that of the arm. If the needle is in and not under the skin, a small whitish bleb comes to view during the injection. Care should be taken to prevent a subcutaneous injection. The areas of injection should be four or five centimeters apart, the tuberculin area being proximal. Reactions as a rule are larger than those in the cutaneous test. Careful observations should be made at 24 and 48 hours and the extent of redness and induration measured. If the patient does not react in four days, a second test may be performed using the next higher dilution: 0.1 cc. = 1.0 mgm. Broth control should be injected in similar dilution with a separate syringe. If one syringe be used, the control injection should be made first and the instrument washed before the tuberculin is injected.

d. *Interpretation and appraisal:* Although in general the tuberculin reaction by no means assumes a major rôle in the diagnosis of tuberculosis, there are, as has already been intimated, several circumstances in which this, and to a less extent, the cutaneous test may be of decided value especially when applied to children. These circumstances occur chiefly in children with (1) equivocal signs of tuberculosis of the tracheobronchial lymph nodes; (2) probable but indefinite signs of tuberculosis of bone or joint; (3) history suggestive of tuberculosis or of intimate exposure to it and with equivocal or negative findings and (4) vague disease of the eye or history of such disease recently. The set of particular circumstances in either of these groups may be such that the tuberculin reaction, although never of itself decisive, may materially help in settling the issue of the diagnosis. For instance in a child with moderate enlargement of the tracheobronchial lymph nodes and a few vague symptoms of being below par, of indisposition to play or of undue fatigue, there would be a good deal of evidence to justify a diagnosis of tuberculosis. But if the intracutaneous test were *consistently negative* and the ordinary causes that negate a reaction be eliminated, one would at least hesitate to make such a diagnosis and would either defer diagnosis and keep the child under observation or arrive at a diagnosis of "no tuberculosis." Occasionally in orthopaedic or ophthalmological practice, a tuberculin reaction may also be extremely helpful, although it is more common to resort to the subcutaneous test in these conditions. In evaluating the status of the undernourished children often seen in tuberculous homes, it is much better to restore the child to a state of normal nutrition before applying the test. As Happ and Casparis (81) have shown, the intracutaneous test frequently causes a reaction in miliary tuberculosis when the Pirquet test fails to do so.

There may be said to be two *disadvantages* to this test. One is the possibility of a subcutaneous injection which might initiate severe reaction. A second disadvantage lies in the delicacy of the test. A procedure yielding so high an incidence of reaction as this one does loses several advantages of a cruder test, although for determining the actual incidence of infection among the generality of people, it is the test of preference.

3. *Subcutaneous test:* This test has no justification as a routine for determining infection because both the tests just described and several others determine that point with greater comfort to the patient and expenditure of less effort and time by the doctor. Occasionally the test is administered in suspected pulmonary tuberculosis. When this is done, careful examination for increase of signs (changes in percussion and especially the appearance of râles) in the lungs during the reaction. This test is applied only infrequently and is usually not only not very helpful but may be and sometimes is dangerous to the patient. The aim in the test should be to get focal reactions, and the test should be limited as much as possible to extra-pulmonary or extra-visceral disease. The rule is, however, to get a general as well as a focal reaction and the symptoms of this are watched for very closely. Its chief use is found in orthopaedic practice where a focal reaction may aid materially in diagnosing the nature of a disability under question.

a. *Mode of application:* The tuberculin should be given subcutaneously in ascending doses, beginning with one small enough to ordinarily prevent a reaction. If there is no reaction to the first, the second and larger dose should follow at the end of two days, and so a third. Hamman and Wolman recommended for adults the general working scheme of administering 1/5, 1, and 5 mgm. if no reaction follows either of the first two, and for children of eight to fourteen years, 0.1 mgm. initially with 1.0 mgm. as maximum. But dosage in both children and adults should vary a good deal depending especially upon the general physical condition and the extent of the supposed tuberculosis. If a slight reaction occurs, the same sized dose and not a larger one should be repeated. The dilutions may be made according to the scheme mentioned above (p. 142). Injections may be made in many places over the body, but the lumbar, gluteal or triceps regions are the most usual sites.

b. *Results:* The estimation of the test depends upon the symptoms that arise from both the focal and the concomitant general reaction. It is therefore necessary to observe the patient for a few days prior to the test in order to ascertain what his usual focal symptoms are (cough, expectoration, fever, pain, etc., if pulmonary; pain, mobility, etc., if joint). Of especial importance is the daily rise of temperature, for the elevation of temperature after the injection is the most important single symptom of the reaction. It is preferable to have the patient afebrile for several days before testing, but an indefinite postponement of the test is not necessary because the patient has fever. Temperature, pulse rate and respiration should be made every two hours for two or three days prior and subsequent to the test.

The response begins to manifest itself six or eight hours after the injection, and it is advisable therefore to inject the tuberculin at 11 or 12 o'clock at night so that one may six hours later begin to observe closely the temperature, pulse and respiratory rate every two hours and look for any change in focal or general symptoms. The reaction has pretty well subsided by 24 hours. It is positive if the temperature has gone a degree above the daily level for the patient. The changes in focal symptoms such as tenderness, swelling or redness of a joint, increase in pain or lessening of mobility, when they occur, are very helpful in confirming the diagnosis. If the focal area be in the eye, the skin or other visible situation, the focal reaction may usually be seen.

c. *Appraisal:* The test is useful in confirming a suggestive diagnosis especially in tuberculosis of bone or joint, but it is of little value in pulmonary disease.

d. *Dangers:* The possibility always and the probability at times of danger from an intense focal reaction is to be borne in mind. The perifocal inflammation may soften the fibrous wall so that absorption by the circulation of soluble substances and tubercle bacilli from the interior of the focus will occur. An extension of the disease may occur by spread of bacilli locally or even generally. Besides this danger, extreme reactions ought always to be avoided when possible because of the general effects of such on the patient.

4. *Other cutaneous tests:* a. *Percutaneous test of Moro (154):* This test consists of rubbing into the skin a solution of tuberculin made of equal parts of Old Tuberculin and lanolin thoroughly mixed together. Results vary, depending upon the manner and length of time of rubbing and the condition of the skin. It yields no information that other tests with less chance of error do not yield.

b. *Subcutaneous-local test:* Hamburger (79) introduced the idea of doing a subcutaneous injection so closely under the skin as to get a subcutaneous injection with local signs of a cutaneous test. The test is very sensitive and has but little value in persons above the age of two or three years. In children below that age it will occasionally bring out a reaction that the cutaneous test has failed to produce.

The Conjunctival Test

This test is performed by instilling a drop of a one per cent dilution of Old Tuberculin in the eye. Calling forth a reaction less readily of any other tests, the reaction is more likely to occur only in persons with active tuberculosis, but there are so many exceptions as to render it of not much importance.

SUMMARY

It must be emphasized again that too much has been expected from tuberculin. The product has a place, occasionally an important place, in diagnosis, but its applications are definitely limited and these limitations should be recognized and understood. When the reaction occurs, it is an infallible sign of tuberculous infection but it gives no reliable cue as to whether tuberculous infection is synonymous with tuberculous disease in the person tested. By using the test only when it is indicated and interpreting its results only in the light of the fact that it never of itself can render a diagnosis but must always be interpreted as an accessory in the thorough appraisal of a given case, it will at times be of considerable value in diagnosis. Otherwise it will have little or no diagnostic worth.[16]

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[16] I have frequently consulted the valuable book of Hamman and Wolman (80) in the preparation of this chapter.

PART IV

SEROLOGICAL DIAGNOSIS

SEROLOGICAL DIAGNOSIS

No constituent of the body has been so much studied for the secrets of the phenomena of disease and none subjected to so many tests for the diagnosis of disease as the serum. Many reactions based upon precipitation and agglutination—the mallein reaction in glanders, the Widal reaction in typhoid fever, the several reactions for typing pneumococci, the Abderhalden reaction—various aggressin reactions and colorimetric tests of several kinds, tests for anaphylactic phenomena, and, as important as any, complement fixation: these are some of the serological tests that have been designed to give aid in the diagnosis of disease.

Many of the serological tests have been applied to tuberculosis as to other diseases, and it is the aim of this section of the book to present several of these to which a great deal of attention has been given and on which much work has been done. Of these tests in tuberculosis, that of complement fixation doubtless stands first. Applied to tuberculosis with several different types of antigen and technical procedure, the test yielded results that were contradictory and equivocal for a good many years. But it appears that now the reaction can be carried out according to one or two standard procedures in such a way as to probably be of some definite (accessory) help in the diagnosis. Dr. J. S. Woolley, who has done a great deal of valuable work with this test, has kindly agreed to write the chapter on complement fixation. An appraisal of this test will be included in his treatment of the subject. The other several tests to be described will be evaluated at the conclusion of the section devoted to serological diagnosis.

CHAPTER X

TUBERCULO-COMPLEMENT FIXATION[17]

by

J. STANLEY WOOLLEY
Loomis Sanatorium, Loomis, N. Y.

GENERAL REMARKS ON COMPLEMENT FIXATION

The principles underlying the complement fixation reaction are now well known and it does not seem necessary to do more than briefly call attention to them. Two methods for determining the presence or absence of this reaction in tuberculosis serum will be described in detail.

If a certain foreign material—protein, bacteria, foreign blood cells, etc.—be inoculated into an animal, substances are formed in the animal's serum which will combine very rapidly with like material when introduced a second time. The material introduced is known as *antigen* and the substance which is formed as the result of the introduction of the antigen is called *amboceptor* (antibody, reagin). The latter is resistant and thermostable and may be formed in the blood as indicated or as the result of an infection, in which case the bacteria causing the infection constitute the antigen. Amboceptor exists, therefore, only in immune serum. The striking characteristic of amboceptor is its strong combining affinity for antigen. It is always specific, for it combines only with that antigen which calls forth its formation. As a result of this combination the antigen is said to be "sensitized."

Complement is a component of all fresh serum whether normal or immune. It is thermolabile, being easily destroyed by heat, and disappears from the serum in a few days at ordinary room temperature. It is non-specific and may be added to or removed from serum at will. The removal of complement from serum is termed "inactivation." This is done by heating the serum. Complement will not combine with antigen or amboceptor alone but readily unites with or fixes itself to sensitized antigen. Lysis of the antigen usually results and simultaneously the complement disappears from the mixture. It is not easy to demonstrate lysis in the case of bacterial or similar antigens, but if erythrocytes be employed, the resulting hemolysis is easily recognized.

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[17] The tables in this chapter were kindly prepared by Mr. F. G. Petrik of the Loomis Laboratory.

These factors are utilized to determine the presence or absence of amboceptor in a given serum for, if present, the addition of a suitable antigen plus complement results in the disappearance of the complement as union between antigen and antibody takes place. The complement is thus used up, "fixed," or removed from its free state in the mixture and is no longer available for union when a second sensitized antigen is introduced in the solution. This principle has long been utilized in the Wassermann reaction as an aid in the diagnosis of syphilis.[18] In this disease, as in tuberculosis, glanders, chronic gonorrhea, etc., specific amboceptor (antibody) is formed in the blood which will unite with its special antigen. For convenience, this amboceptor or antibody is termed "reagin," following the nomenclature of Neisser. The serum to be tested for the presence of "reagin" is heated to destroy any complement present and is then incubated in a solution containing known amounts of antigen and complement (guinea pig serum). This may be called the "serum antigen" system. If the serum contains a sufficient amount of reagin it unites with the antigen, using up or "fixing" the complement in the process. This, however, cannot be detected without the addition of an indicator which generally consists of a suspension of sheep red cells sensitized by treatment with the serum of rabbits immune to sheep cells (specific amboceptor). This sensitizing serum is usually referred to as "hemolysin." The indicator is called the "hemolytic system" and only requires the presence of free complement to bring about the hemolysis of the red cells. If all the complement has been used up or fixed in the "serum-antigen" system, no reaction takes place when the "hemolytic" system is added, for reagin is present and the test is positive. If there is no reagin present in the serum, complement is not fixed and is left free to act when the hemolytic system is added. This results in the hemolysis of the sheep cells and the test is negative.

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[18] For a more extended discussion of the theory and application of the complement-fixation test, the reader is referred to such standard works as Park, Williams and Krumwiede (170) or Zinsser (243).

The principles underlying the application of the complement fixation reaction to tuberculosis for the detection of specific reagin in the blood are fundamentally the same as in the Wassermann test (225) except that biologically the reaction is truly more specific, for in the Wassermann test a non-specific lipoid antigen has superseded the use of antigen made from syphilitic material. As the concentration of the antibodies or reagin in the blood in tuberculosis is never as great as in frank syphilis, the use of larger amounts of serum than ordinarily employed in the Wassermann test is advised. The reagents must be prepared with meticulous care and careful attention must be given to the details of the technique. Non-specific fixation is more often met with in tuberculo-fixation than in the Wassermann test as approximately 10 per cent of normal or non-tuberculous individuals give slight inhibition of hemolysis. Hence it is usual to disregard weak fixation when the test is used for diagnostic purposes in tuberculosis. Cross-fixation (false positive) does occasionally occur with the sera of certain syphilitics, when a lipoid containing antigen is used in the tuberculo-fixation, the lipoids of such an antigen apparently combining with syphilitic reagin to fix complement. Under these circumstances it is advisable that every serum be given a parallel Wassermann test, and a positive tuberculosis finding in any Wassermann reacting serum should always be interpreted with great caution. To avoid this difficulty the use of a "defatted" or lipoid free antigen has been suggested for tuberculo-fixation, but there is some question as to the value of antigens made from defatted bacilli. They are not as sensitive as those made from whole bacilli.

The serum of tuberculous patients occasionally gives a false positive Wassermann reaction with a cholesterinized antigen. Such cross-fixation may be avoided by the use of a plain alcoholic-extract antigen in the Wassermann technique. The Kolmer technique rarely gives such cross fixation.

The PETROFF TECHNIQUE (177) has had extended usage and will be described; also the LOOMIS-KOLMER TECHNIQUE (Woolley and Petrik (237)). The latter is recommended for workers acquainted with the Kolmer Wassermann technique as it is really an adaptation of this technique to tuberculo-fixation. The combination works well when a Wassermann test is being done on each serum. Following a description of materials and reagents used in one or both of the techniques, the titrations and set up for the tests will be given in table form as this has been found most practical for the average worker. Explanatory notes will accompany the tables whenever necessary.

The Petroff technique utilizes in the hemolytic system the varying amounts of natural anti-sheep hemolysin usually present in human blood-serum. The Loomis-Kolmer technique provides for such hemolysin by the use of a large excess of amboceptor, for, with this excess the addition of a unit or so of natural hemolysin does not affect the unit of complement. The employment of a suitable excess of amboceptor is believed also to result in the dissociation of much nonspecifically bound complement which is a valuable feature of this technique.

The antigens employed in complement fixation in tuberculosis have included simple saline suspensions of tubercle bacilli, alive, autolysed or heated; glycerine-water bacillary extracts; alcoholic and sodium hydroxide extracts; tuberculins and other culture filtrates, and antigens consisting of both bacilli and the medium on which they are grown. Recently, Wassermann (226) has introduced a "defatted" antigen made from bacilli extracted with a special fat solvent, *tetraline*, and enriched by the addition of a small amount of lecithin. Wassermann claimed a strict specificity for his technique. His work remains to be corroborated. For general use, boiled extracts of whole tubercle bacilli have given superior results and are easy to prepare. The presence of lipoids in such antigens aids in the dispersion of the antigenic material, resulting in a smooth and sensitive antigen.

Preparation of Materials and Reagents

1. *Glassware:* All glassware used in the test should be dry, sterile and chemically clean. The test tubes are best prepared by boiling in soapy water and rinsing thoroughly. They are then soaked in 1 per cent hydrochloric acid over night, rinsed again to remove the acid, washed once with distilled water, inverted and dry sterilized. Tubes measuring 0.5 by 4 inches will be found satisfactory, and pipettes having widely spaced graduations are recommended.

2. *Saline solution:* The distilled water used should not be more than two or three days old. It is brought to a boil and immediately cooled in tap water. Eight and one-half grams of sodium chloride, c.p., are added to one liter of the cooled distilled water, neutralized to pH 7.2–7.5, and sterilized in an "Arnold" for one hour. Ordinary distilled water may have an acidity as high as pH 6, and it is very important to have the salt solution properly adjusted if reliable results are to be obtained.

3. *Complement:* The blood of four or more normal guinea pigs of 500 to 700 gm. weight (which have fasted for 18 to 24 hours) is collected in sterile, chemically clean, 15 cc. centrifuge tubes. It is allowed to stand at room temperature for one or two hours, then in the ice-box over night, and is finally centrifugalized at high speed. Several methods may be used for obtaining the blood. The following is an economical procedure for collecting large quantities. The skin above the external jugular vein is shaved, while dry, and cocainized, and the hind legs are fastened to a ring on a stand, with the body, head downward, held by an assistant. A small oblique incision, about one centimeter long, is made directly over the jugular; and the vein, exposed by separating the tissues with a blunt scissors, is cut, and the blood allowed to drip directly into a centrifuge tube. A piece of cotton is then pressed against the incision until the bleeding stops, and one stitch is made to close the wound. No special aseptic precautions are necessary. In this manner 7 to 9 cc. of blood may be obtained and the

guinea pigs can be bled four times, but not oftener than once every three weeks. After the fourth bleeding they can be used for routine purposes. It is now well known that the serum of an occasional guinea pig is unsuitable for the fixation test for tuberculosis because such complement fails to fix with the serum of tuberculous individuals. There is apparently no definite relationship between the activity of complement and the fixability of a given specimen of guinea pig serum; hence the importance of using a pooled complement as described. The complement is best used fresh, if possible. It will keep well for forty-eight hours on the clot. It is diluted 1–10 (1 cc. of complement to 9 cc. of the saline solution) for use in the Petroff technique and 1–30 for the Loomis-Kolmer method.

4. *Hemolysin:* This is prepared by injecting washed sheep red corpuscles into rabbits. Any of the well known methods may be used but only those showing a high titer should be employed in the Loomis-Kolmer technique. The antisheep (rabbit) hemolysin prepared by the H. K. Mulford Company has been found satisfactory. For the Petroff technique the hemolysin is diluted with the saline solution so that 0.2 cc. represents two units, as will be seen in the tables. The Loomis-Kolmer technique employs a much stronger solution of amboceptor (10 units) for testing the patient's serum.

5. *Red cells:* Fresh sheep blood is defibrinated with glass beads or broken glass rods and then washed at least six times with saline solution. A 5 per cent solution is used for the Petroff test and a 2 per cent in the Loomis-Kolmer technique. Blood preserved with formalin has not yielded satisfactory results in our hands.

6. *Patient's serum:* The blood is collected in the usual manner and is allowed to remain on the clot over night. By centrifugalizing the tubes after separating the clot, a maximum amount of serum may be obtained. For the Petroff technique the serum is inactivated before testing for one-half an hour in the water-bath at 56°C. The Loomis-Kolmer technique requires the same inactivation as the Kolmer Wassermann technique, namely 15 minutes in the water-bath at 55°C.

7. *Serum for antigen titrations:* a. *Serum for antigenic unit:* A mixture of four or more fresh sera giving four-plus tuberculo-fixation by either technique in 0.1 cc. amounts is used to determine the antigenic unit of the respective antigens.

b. *Standard serum:* The above mixed positive serum is pipetted in 0.1 cc. amounts into small test tubes and stored unheated in a desiccator over sulphuric acid. Before use, 0.1 cc. of distilled water is added to each tube and the mixture thoroughly shaken until the serum is dissolved. The resulting solution is slightly milky. The use of such serum increases the titer of the antigen about 50 per cent, the increase being largely non-specific. However, the fixing power of the dried serum remains constant for months and may be used to keep a check on the stability of the antigen. Such dried positive serum rarely becomes anticomplementary.

8. *Antigen for the Petroff method* (*Petroff's New Antigen*): Tubercle bacilli are grown on 4 per cent glycerine beef broth for from four to six weeks, and the whole contents of the culture is filtered through several layers of filter paper. The bacilli are then washed with normal salt solution until the filtrate does not give a biuret reaction. They are next spread out in a thin layer in a flat dish and dried in a desiccator over sulphuric acid. This usually takes about a week. Five grams of the dried bacilli are then pulverized by grinding in a ball mill for two weeks, the mill not making more than 60 revolutions per minute. The ground mass is now extracted with 100 cc. of pure toluol at 37.5°C. for five days. The extract and residue are then evaporated to dryness, in a well ventilated incubator, with the aid of a fan. The remaining dried mass is then returned to the mill, 20 cc. of 25 per cent glycerine is added and the mixture ground in mill for 48 hours. It is then transferred with a pipette to a liter-flask and the porcelain mill jar washed out with the same solution, the washings and sufficient 25 per cent glycerine being added to make a total of 500 cc. in the flask. This is boiled gently for one hour with a reflux condenser, allowed to stand for three or four hours to facilitate the settling out of the large clumps. The solution is then transferred to suitable receptacles without disturbing the settled clumps. The antigen

should be kept on ice a week before using. If stored in a refrigerator, it will keep a year without becoming anticomplementary.

The test dose should not be more than one-fourth the anticomplementary unit; with a sensitive antigen, ordinarily one-sixth of the unit is employed (for explanation of units, see tables).

9. *Antigen for the Loomis-Kolmer method:* The following simple antigen has been found to give satisfactory results. One gram of dried human tubercle bacilli is ground by hand in a mortar (under a hood) for one-half hour, distilled water being added drop by drop until a smooth suspension is obtained. This is then diluted with distilled water to 100 cc., transferred to a small flask (250 cc.) fitted with a reflux condenser and boiled as gently as possible for two hours. The "low heat" of a small electric stove will usually be more satisfactory than a flame, and three or four drops of caprylic alcohol should be added to prevent foaming. If there are no clumps, the suspension is then pipetted into 25 cc. pyrex or non-sol flasks, stoppered with cotton and heated in a slowly boiling water-bath for another hour to drive off the caprylic alcohol and to further insure sterility. The flasks are then corked with sterile rubber stoppers and later sealed with wax. If clumps are present, the extract is allowed to stand at room temperature for three or four hours until the larger clumps settle out. The whole process had best be carried on under a hood. The completed antigen is allowed to ripen for at least two weeks at ordinary ice-box temperature before using. It will keep for from four to six months if stored at a low temperature.

The test dose should not be more than one-fourth the anticomplementary unit and should contain from five to seven antigenic units (see tables for units). It is only rarely that the above antigen is hemolytic in any strength. The set-up for the hemolytic titration is given in table 15. When hemolysis is present, the smallest amount of antigen just beginning to show this is called the hemolytic unit. The test dose should not be stronger than one-sixth this amount.

TABLE 8 (Petroff Technique)

AMBOCEPTOR TITRATION

Tube No.	Complement 1 Unit	Amboceptor 1–1000	Red Cells 5 %	Salt Solution 0.85 %		Results
	cc.	cc.	cc.	cc.		
1	0.1	0.02	0.2	1.18	Incubate in water bath for 30 minutes at 38°C	No hemolysis
2	0.1	0.05	0.2	1.15		Moderate hemolysis
3	0.1	0.08	0.2	1.12		Marked hemolysis
4	0.1	0.10	0.2	1.10		*Complete hemolysis (unit)*
5	0.1	0.12	0.2	1.08		Complete hemolysis
6	0.1	0.15	0.2	1.05		Complete hemolysis
7	0.1	0.18	0.2	1.02		Complete hemolysis
8	0.1	0.20	0.2	1.00		Complete hemolysis

In instituting the test the amboceptor may be titred with 0.1 cc. of a 10 % solution of complement as an arbitrary unit.

The first tube showing complete hemolysis is the unit. Two units are used in the test.

The original Petroff technique calls for 0.25 cc. of 5 % red cells throughout the test. We were never able to obtain complete hemolysis with this amount hence reduced the quantity to 0.2 cc. with satisfactory results.

TABLE 9 (Petroff Technique)

COMPLEMENT TITRATION

Tube No.	Complement 10 %	Amboceptor 2 Units	Red Cells 5 %	Salt Solution 0.85 %		Results
	cc.	cc.	cc.	cc.		
1	0.05	0.2	0.2	1.05	Incubate in water bath for 30 minutes at 38°C.	No hemolysis
2	0.07	0.2	0.2	1.03		Slight hemolysis
3	0.08	0.2	0.2	1.02		Moderate hemolysis
4	0.09	0.2	0.2	1.01		Marked hemolysis
5	0.1	0.2	0.2	1.00		*Complete hemolysis (unit)*
6	0.12	0.2	0.2	0.98		Complete hemolysis
7	0.13	0.2	0.2	0.97		Complete hemolysis
8	0.15	0.2	0.2	0.95		Complete hemolysis
9	0.2	0.2	0.2	0.90		Complete hemolysis

Two units are used in the test; 0.2 cc. therefore would contain two units.

TABLE 10 (Petroff Technique)

ANTICOMPLEMENTARY TITRATION OF ANTIGEN

Tube No.	Antigen 1–30	Complement 2 Units	Salt Solution 0.85%		Amboceptor 2 Units	Red Cells 5%		Results
	cc.	cc.	cc.		cc.	cc.		
1	0.1	0.2	0.7	Incubate in water bath for 1½ hours at 38°C.	0.2	0.2	Incubate in water bath for 30 minutes at 38°C.	No inhibition of hemolysis
2	0.2	0.2	0.6		0.2	0.2		No inhibition of hemolysis
3	0.3	0.2	0.5		0.2	0.2		No inhibition of hemolysis
4	0.4	0.2	0.4		0.2	0.2		No inhibition of hemolysis
5	0.5	0.2	0.3		0.2	0.2		No inhibition of hemolysis
6	0.6	0.2	0.2		0.2	0.2		*Slight inhibition of hemolysis (unit)*
7	0.7	0.2	0.1		0.2	0.2		Moderate inhibition of hemolysis
8	0.8	0.2	—		0.2	0.2		Marked inhibition of hemolysis
9	0.9	0.2	—		0.2	0.2		Complete inhibition of hemolysis
10	1.0	0.2	—		0.2	0.2		Complete inhibition of hemolysis

The first tube showing slight inhibition of hemolysis is called the anticomplementary unit. The antigen is so diluted that 0.1 cc., the dose used in the test, is one-sixth the anticomplementary unit.

Table 11 (Petroff Technique)

ANTIGENIC TITRATION OF ANTIGEN

Tube No.	4 Plus Tuberculosis Serum	Antigen 1–30	Complement 2 units	Salt Solution 0.85 %		Amboceptor 2 units	Red cells 5 %		Results
	cc.	cc.	cc.	cc.		cc.	cc.		
1	0.1	0.01	0.2	0.69	Incubate in water-bath for 1½ hours at 38°C.	0.2	0.2	Incubate in water-bath for 30 minutes at 38°C.	Moderate inhibition of hemolysis
2	0.1	0.02	0.2	0.68		0.2	0.2		*Complete inhibition of hemolysis* (unit)
3	0.1	0.03	0.2	0.67		0.2	0.2		Complete inhibition of hemolysis
4	0.1	0.05	0.2	0.65		0.2	0.2		Complete inhibition of hemolysis
5	0.1	0.08	0.2	0.62		0.2	0.2		Complete inhibition of hemolysis
6	0.1	0.10	0.2	0.60		0.2	0.2		Complete inhibition of hemolysis
7	0.1	0.15	0.2	0.55		0.2	0.2		Complete inhibition of hemolysis
8	0.1	0.20	0.2	0.50		0.2	0.2		Complete inhibition of hemolysis
9	0.1	—	0.2	0.70		0.2	0.2		No inhibition of hemolysis

This titration gives an index of the fixing quality of the antigen. The unit is usually the 0.02 cc. tube so that in 0.1 cc. we have 5 antigenic units and this amount is one-sixth the anticomplementary unit. Tube No. 9 is for the anticomplementary control of the serum.

TABLE 12 (Petroff Technique)

THE TEST

Tube No.	Patients Serum	Antigen 1—30	Complement 2 Units	Salt Solution 0.85%		Red cells 5%		Amboceptor 2 Units	Red cells 5%		Result
	cc.	cc.	cc.	cc.	Incubate in water-bath for 1 1/2 hours at 38°C.	cc.	Incubate in water-bath for fifteen minutes at 38°C.	Units	cc.	Incubate in water in bath for 30 minutes at 38°C.	
1	0.1	0.1	0.2	0.6		—		2	0.2		*Marked inhibition of hemolysis if positive*
2	0.1	—	0.2	0.7		0.2		2	—		No inhibition of hemolysis
3	—	0.2	0.2	0.6		—		2	0.2		No inhibition of hemolysis
4	—	0.3	0.2	0.5		—		2	0.2		No inhibition of hemolysis
5	—	0.4	0.2	0.4		—		2	0.2		No inhibition of hemolysis
6	—	0.5	0.2	0.3		—		2	0.2		No inhibition of hemolysis
7	—	0.6	0.2	0.2		—		2	0.2		Slight inhibition of hemolysis

If hemolysis is complete in tube No. 2 do not add any amboceptor to this tube or tube No. 1. If partial hemolysis is present in No. 2 add 0.1 cc. (1 unit) of amboceptor to tubes No. 1 and 2. If no hemolysis is present, add 0.2 cc. (2 units) of amboceptor to tubes No. 1 and 2. Tubes Nos. 3, 4, 5, 6 and 7 always receive 0.2 cc. (2 units) of amboceptor. These tubes are for the anticomplementary control of the antigen. One set of controls is set up with each series of tests.

PETROFF TECHNIQUE

Results are read directly and after standing over-night in the ice-box. Only 3 plus and 4 plus fixations are considered positive.

Table 13 (Loomis-Kolmer Technique)

AMBOCEPTOR TITRATION

Number	Complement 1–30	Amboceptor 0.5 cc.	Red cells 2 per cent	Salt Solution 0.85 per cent	Incubate for 45 minutes in water-bath at 38°C.	Results
	cc.		cc.	cc.		
1	0.3	1–10,000	0.5	1.7		Slight hemolysis
2	0.3	1– 9,000	0.5	1.7		Moderate hemolysis
3	0.3	1– 8,000	0.5	1.7		*Complete hemolysis (Unit)*
4	0.3	1– 7,000	0.5	1.7		Complete hemolysis
5	0.3	1– 6,000	0.5	1.7		Complete hemolysis
6	0.3	1– 5,000	0.5	1.7		Complete hemolysis
7	0.3	1– 4,000	0.5	1.7		Complete hemolysis
8	0.3	1– 3,000	0.5	1.7		Complete hemolysis
9	0.3	1– 2,000	0.5	1.7		Complete hemolysis
10	0.3	1– 1,000	0.5	1.7		Complete hemolysis

Table 14 (Loomis-Kolmer Technique)

COMPLEMENT TITRATION

Tube number	Complement 1–30	Antigen 5–7 Units	Salt solution 0.85 per cent	Incubate for one hour in water-bath at 38°C.	Amboceptor 10 Units	Red cells 2 per cent	Incubate for 45 minutes in water-bath at 38°C.	Results
	cc.	cc.	cc.		cc.	cc.		
1	0.65	0.5	0.85		0.5	0.5		Complete hemolysis
2	0.60	0.5	0.90		0.5	0.5		Complete hemolysis
3	0.55	0.5	0.95		0.5	0.5		Complete hemolysis
4	0.50	0.5	1.00		0.5	0.5		Complete hemolysis
5	0.45	0.5	1.05		0.5	0.5		Complete hemolysis
6	0.40	0.5	1.10		0.5	0.5		_Complete hemolysis (Unit)_*
7	0.35	0.5	1.15		0.5	0.5		Marked hemolysis
8	0.30	0.5	1.20		0.5	0.5		Moderate hemolysis
9	0.25	0.5	1.25		0.5	0.5		Slight hemolysis
10	0.20	0.5	1.30		0.5	0.5		No hemolysis
11	2.00	Omit	0.50		Omit	0.5		(Hemolytic complement control) No hemolysis

* Two units are used in the test; 0.8 cc. therefore would contain two units. The complement is then diluted so that 1cc. = 2 units. For example: 2 units:30 ::1:x. x = dilution to be used in the test and 1cc. contains two units.

Table 15 (Loomis-Kolmer Technique)

HEMOLYTIC TITRATION OF ANTIGEN

Tube Number	Antigen 0.5 cc.	Red cells 2 per cent	Salt solution 0.85 percent	Incubate for one hour in water-bath at 38°C.	Results
		cc.	cc.		
1	Full strength	0.5	2.0		No hemolysis*
2	1–2	0.5	2.0		No hemolysis
3	1–3	0.5	2.0		No hemolysis
4	1–4	0.5	2.0		No hemolysis

* Tubes containing large amounts of antigen should be centrifugated to determine any hemolysis.

TABLE 16 (Loomis-Kolmer Technique)

ANTICOMPLEMENTARY TITRATION OF ANTIGEN

Tube number	Antigen 0.5 cc.	Complement 2 units	Salt solution 0.85 per cent	Incubate for two hours in water-bath at 38°C.	Amboceptor 10 units	Red cells 2 per cent	Incubate for 45 minutes in water-bath at 38° C.	Results
		cc.	cc.		cc.	cc.		
1	Full strength	1.0	0.5		0.5	0.5		Complete inhibition of hemolysis
2	1–2	1.0	0.5		0.5	0.5		Complete inhibition of hemolysis
3	1–3	1.0	0.5		0.5	0.5		Moderate inhibition of hemolysis
4	1–4	1.0	0.5		0.5	0.5		Moderate inhibition of hemolysis
5	1–5	1.0	0.5		0.5	0.5		*Slight inhibition of hemolysis (Unit)**
6	1–6	1.0	0.5		0.5	0.5		No inhibition of hemolysis
7	1–7	1.0	0.5		0.5	0.5		No inhibition of hemolysis
8	1–8	1.0	0.5		0.5	0.5		No inhibition of hemolysis
9	1–9	1.0	0.5		0.5	0.5		No inhibition of hemolysis
10	1–10	1.0	0.5		0.5	0.5		No inhibition of hemolysis

* Tubes containing large amounts of antigen should always be centrifugated to determine any inhibition of hemolysis.

TABLE 17 (Loomis-Kolmer Technique)

DIRECTIONS FOR DILUTING THE ANTIGEN FOR ANTIGENIC TITRATIONS

0.2 cc. of 1–4* dilution plus 1.8 cc. of saline solution	1–40
0.2 cc. of 1–6 dilution plus 1.8 cc. of saline solution	1–60
0.2 cc. of 1–8 dilution plus 1.8 cc. of saline solution	1–80
0.2 cc. of 1–10 dilution plus 1.8 cc. of saline solution	1–100
0.2 cc. of 1–12 dilution plus 1.8 cc. of saline solution	1–120
1.0 cc. of 1–80 dilution plus 1.0 cc. of saline solution	1–160
1.0 cc. of 1–100 dilution plus 1.0 cc. of saline solution	1–200
1.0 cc. of 1–120 dilution plus 1.0 cc. of saline solution	1–240
1.0 cc. of 1–160 dilution plus 1.0 cc. of saline solution	1–320
1.0 cc. of 1–200 dilution plus 1.0 cc. of saline solution	1–400

* One of antigen to three of saline solution, etc. Add antigen to salt drop by drop, constantly agitating.

Table 18 (Loomis-Kolmer Technique)

ANTIGENIC TITRATION OF ANTIGEN

Tube number	4+serum (4 pooled)	Antigen 0.5 cc.		Complement 2 units	Salt solution 0.85 per cent		Amboceptor 10 units	Red cells 2 per cent		Results
	cc.			cc.	cc.		cc.	cc.		
1	0.1	1–400*	Wait 10 minutes	1.0	0.4	Incubate for two hours in water-bath at 38°C.	0.5	0.5	Incubate for 45 minutes in water-bath at 38°C.	Complete hemolysis
2	0.1	1–320		1.0	0.4		0.5	0.5		Slight inhibition of hemolysis
3	0.1	1–240		1.0	0.4		0.5	0.5		Moderate inhibition of hemolysis
4	0.1	1–200		1.0	0.4		0.5	0.5		*Complete inhibition of hemolysis* (*Unit*)
5	0.1	1–160		1.0	0.4		0.5	0.5		Complete inhibition of hemolysis
6	0.1	1–120		1.0	0.4		0.5	0.5		Complete inhibition of hemolysis
7	0.1	1–100		1.0	0.4		0.5	0.5		Complete inhibition of hemolysis
8	0.1	1–80		1.0	0.4		0.5	0.5		Complete inhibition of hemolysis
9	0.1	1–60		1.0	0.4		0.5	0.5		Complete inhibition of hemolysis
10	0.1	1–40		1.0	0.4		0.5	0.5		Complete inhibition of hemolysis
11†	0.1	Omit		1.0	0.9		0.5	0.5		Complete hemolysis

* The highest dilution of antigen is given first for convenience in pipetting.

† Tube 11 is the anticomplementary control of the serum.

TABLE 19 (Loomis-Kolmer Technique)

THE TEST

Tube number	Patient's serum	Antigen (5–7 units)	Wait 10 minutes	Complement 2 units	Salt solution 0.85 per cent	Incubate for two hours in water-bath at 38°C.	Amboceptor 10 units	Red cells 2 per cent	Incubate for 45 minutes in water-bath at 38°C.	Results
	cc.	cc.		cc.	cc.		cc.	cc.		
1	0.2	0.5		1.0	0.3		0.5	0.5		*Definite inhibition of hemolysis if positive*
2	0.2	Omit		1.0	0.8		0.5	0.5		Complete hemolysis
3	Omit	cc. 0.5		1.0	0.5		0.5	0.5		Complete hemolysis
4	Omit	1.0		1.0	Omit		0.5	0.5		Complete hemolysis
5	Omit	1.5		1.0	Omit		0.5	0.5		Complete hemolysis

Tube 2 is the anticomplementary control of the serum.

Tubes 3, 4 and 5 are for the anticomplementary control of the antigen. One set of these tubes should be included in each series of tests performed.

Recording of Results

Results are read immediately following the secondary incubation and are reported as follows:

$$\left.\begin{array}{l}0\\+\end{array}\right\} = \text{Negative}$$

++ = Doubtful
+++ = Moderately Positive
++++ = Positive
(Loomis-Kolmer Technique)

Explanatory Data

In this technique it will be noted that 0.2 cc. of the patient's serum is used in the test, whereas only 0.1 cc. of positive serum is employed in the antigen titrations. This doubling the amount of serum frequently results in a positive reaction in sera which would be negative or doubtful if tested in 0.1 cc. amounts, due to the low concentration of antibodies present. In the antigenic titration of the antigen, if a strongly positive serum is used, the unit will be practically the same whether 0.2 or 0.1 cc. is used; hence, to make the test as uniform as possible with the Kolmer-Wassermann technique and for reasons of economy, the employment of the smaller amount of serum is recommended. In hundreds of tests using 0.2 cc. of patient's serum, this amount of serum was never found to be anticomplementary.

Extra Tubes

Where it is convenient to do more than a two-tube test the following extra tubes can be used: No. 1 using 0.1 cc. of patient's serum, employing the regular procedure and No. 2 using 0.2 cc. of patient's serum, three units of antigen (0.5 cc. contains 3 units or is half strength), and one unit plus 0.1 cc. of complement (e.g., 0.5 cc. plus 0.1 cc. equal to 0.6 cc.) making up to volume with saline solution. The rest of the procedure is the same as for the other tubes.

Tube No. 1 determines the strongly positive sera and tube No. 2 detects the weak reactors. The very small excess of complement used in tube No. 2 may slightly increase the nonspecific fixation, hence a 2 plus result should be considered negative and a 3 plus a border-line reaction (unless there be very definite confirmation in the other tubes). The use of the two extra tubes, making a four tube test, will be found helpful for routine purposes, as the results have a definite quantitative value. It will be necessary to use tube No. 1 to detect the sera for use in the antigenic titration of antigen (table 18).

In testing cerebrospinal fluid the test must be made as soon as possible on the unheated fluid and much larger amounts employed (0.25, 0.50 and 0.75 cc.) than in the regular test.

The Value of the Tuberculo-Complement Fixation Test

Carefully conducted tests should yield from 60 to 80 per cent positives depending on the stage of the disease. The antibody curve is at first low but rises as the disease progresses and falls rapidly with the onset of terminal symptoms. Up to a certain point this bears a close relationship with the activity of the disease in the sense that activity is a manifestation of absorption from a focus of infection, for, without absorption, reagin would not be formed. Furthermore, there must be an excess of reagin present—more than enough to saturate the circulating antigen. In any individual the presence of a definitely positive reaction would be presumptive evidence of some absorption from a tuberculous focus, but such absorption might be insufficient in amount to give objective or subjective manifestations, namely clinical activity. The persistence of a positive reaction after the disappearance of any signs of clinical activity is more likely to be a response to antigen production, however minute, than to persistence of reagin previously formed. A negative reaction in such an individual could be interpreted as indicating no absorption. In most instances this is probably true but there are occasional exceptions, and the possibility of the presence of some inhibiting substance which interferes with the reaction of fixation cannot altogether be disregarded. In the more advanced cases, with definite and constant activity, the presence of a negative reaction must be due to some other factor than lack of absorption. In such cases it is more likely due to exhaustion of the tissues engaged in antibody formation, or saturation of the antibodies by overproduction of antigen.

The presence of a definitely positive reaction in any individual cannot be lightly disregarded for normal or nontuberculous persons rarely give marked fixation. The presence of such fixation indicates the existence of some focus of tuberculosis which may be entirely latent as far as clinical signs go. On the other hand, a negative reaction in a subject patently ill with symptoms and signs suggesting tuberculosis, has not, from the very nature and limitations of the reaction, the significance of a positive test. Nevertheless, such a negative reaction increases to a very considerable degree the probability of the nontuberculous nature of the case, particularly if the signs and symptoms are of sufficient intensity to warrant the belief that, if of tuberculous origin, they would indicate definite absorption from some lesion.

From what has been said it will be seen that the test is somewhat limited in its application and its findings are not always as decisive as in the Wassermann test. Its greatest value lies in its helpfulness in ruling out tuberculosis in certain pulmonary conditions, that is, in its *negative value*. In dealing with chronic bronchitis, pulmonary fibrosis, early bronchiectasis, and some forms of pulmonary tumor and abscess, it is often difficult to determine whether the lesion may not be one of those bizarre forms of tuberculosis in which there is no sputum, or, if the latter is present, showing no tubercle bacilli. A negative complement-fixation will help to rule out tuberculosis in many of these cases. On the other hand, a definite positive reaction makes the presence of tuberculosis very probable either as the cause or rarely as an accompanying disease, for experience (Loomis-Kolmer technique) has shown that non-tuberculous individuals rarely give the stronger fixations. With regard to syphilis of the lungs, the same rule holds good as applies to syphilis elsewhere in the body and here a positive complement-fixation may be the result of cross-fixation and nothing else.

The test is of little value in determining the amount of *clinical activity* although a persistent and definitely positive reaction usually means active or recently active tuberculosis. The degree of activity and the amount of fixation do not necessarily run parallel in individual cases. In practise it will frequently be found that with an abatement of the signs and symptoms, a previous weak reaction may become strongly positive. This is likely due to a reduction in the amount of circulating antigen resulting in a great excess of unfixed reagin. Also, for similar reasons, advanced cases showing a strongly positive reaction have a better prognosis than those reacting weakly or not at all.

In those cases of *early tuberculosis* with a negative sputum and in all cases of *suspected tuberculosis*, the presence of a definitely positive complement-fixation increases greatly the probability of the correctness of the diagnosis.

The test is of less value in the different forms of *extra-pulmonary* tuberculosis (pleurisy, etc.) as these conditions usually do not show as high a percentage of positives as the pulmonary type of the disease.

CHAPTER XI

OTHER SEROLOGICAL TESTS

A large number of serological reactions for the diagnosis of tuberculosis have been presented from time to time, many of them eventually being consigned to the discard. These, as Pinner (180) has pointed out, fall into three groups, namely; (1) antibody reactions, such as complement deviation, inhibitive reaction of Caulfeild, agglutination and precipitation; (2) reactions designed to demonstrate specific antigens in the serum or the body, such as the auto-serum or auto-urine reactions; and (3) reactions resting on nonspecific changes in the blood but claimed to be characteristic of active or latent stages of tuberculosis. In this latter group fall such tests as the rate of sedimentation of red blood cells, flocculation test for colloidal stability of the serum, etc. Several of these reactions are based upon perfectly rational theoretical consideration but none has proved to be of unerring value and very few have any dependable worth. Even when the better ones are of aid, they must be regarded as corroborative or accessory aids and interpreted only in this light. One or two of them appear to possess some prognostic value, as will be indicated later. A few of these reactions will be briefly described.

INHIBITIVE REACTION

Tuberculo-complement fixation has already been described in Chapter X. It is in association with this reaction that Caulfeild (35) has discovered a substance, frequently present in sera of people with tuberculosis, which prevents antigen from fixing complement. In the test, serum is added to tubes containing three-multiple dilutions of tuberculo-lypoidal antigen. In the terms of Caulfeild the latter is "digested" by the serum, "the degree of the 'digestion' being shown by the extent to which the anticomplementary strength of the antigen is destroyed." According to Caulfeild, positive results (haemolysis after addition of the haemolytic system) in general are found in cases with clinical activity and indicate a favorable prognosis, yet positive instances do sometimes occur in which no signs of disease are clinically discoverable. He claimed that serial tests are particularly helpful, for the reaction may vary considerably even at short intervals. Those supporting the theory stated that the occurrence of an unexpected positive result in a previously negative patient indicates that activation will likely appear shortly. In the terms of the author the test is rarely positive in normal people, and its presence should cause suspicion of subsequent appearance of tuberculosis.

Perla (175)(176) studied the reaction and found that, although often present in tuberculosis, as Caulfeild recorded, it is not specific for it can be elicited when, instead of serum, gelatin, gumarabic, egg albumin, dextrine, serum albumin or globulin, or diptheria antitoxin is added to the tuberculous antigen. He also showed that it may be demonstrated in any nonspecific alcoholic lipoidal solution as well as in antigen prepared from tubercle bacilli. The reaction depends upon an increase of the "protective colloidal constituents in the serum," and may be obtained in a high percentage of clinically nontuberculous patients.

"Antibody" and "Immunebody" Reactions

As is well known, a considerable number of substances have been described in the serum of sick persons under the generic terms—"antibodies" or "immune bodies," the demonstration of which has been used as a diagnostic criterion. Several of them differ in principle, but for the present purpose may be considered together. The chief ones, agglutinins and precipitins have been studied very extensively in tuberculosis and both have gone into the discard in so far as dependence upon them for diagnosis is concerned.

It is easy, by inoculation of tubercle bacilli, to produce substances in the blood of animals that will agglutinate these microörganisms if the latter are brought into contact with the serum. It is likewise an easy matter to add to such sera a little tuberculin and obtain a precipitation. But neither reaction is specific since a fairly large number of normal, healthy people give one or both of the reactions as do those ill with other diseases than tuberculosis. Therefore, they cannot be depended upon as diagnostic procedures. And it may be remarked by way of parenthesis that, although they have frequently been held to indicate immunity to tuberculosis, this cannot be the case because the reaction is sometimes present and sometimes absent in man and animals that are immune and likewise sometimes present and sometimes absent in those that are not immune. Attention has been called to the fact that "cross-agglutination," "cross-precipitation" etc. can be produced with the non-pathogenic acid-fast bacilli (p. 126). These reactions, however, have no diagnostic or immunological importance.

Opsonic Index

It has been observed that leucocytes appear to possess no capacity of phagocytosis except they be in contact with serum. In the presence of the latter, however, they do have the power to ingest and probably destroy bacteria. It appears that a substance in the serum acts upon bacteria in such a way as to "prepare" them for phagocytosis: it was to this substance that Wright applied the term "opsonin" more than 20 years ago. In animals made immune to certain specific diseases, phagocytosis of the causative bacterium procedes at a remarkably accelerated pace, and the degree of this increased capacity is represented by the *opsonic index* which is simply the ratio of the number of bacteria ingested per leucocyte from immune serum to the number of the same bacteria ingested per leucocyte in serum from a normal animal, when the bacteria are added similarly to preparations of leucocytes from each of these sources. The technique is very refined and involved and is one in which only considerable experience and care will prevent error.

Although in a good many diseases the determination of the opsonic index may have considerable value, it has no such value in tuberculosis. A great deal of work has been done both in tuberculous animals and man, but, as Baldwin (9) pointed out in a detailed study of the subject, great inconstancy features the results. Single observations on an individual are worthless. It may be, as Calmette (31) stated, that, provided it could be "performed and reperformed by the same observer," the test might aid to some extent in *prognosis*, but as a diagnostic procedure its value is nil.

TESTS FOR SPECIFIC ANTIGENS

Numerous diagnostic tests of this type, the most widely known of which in recent years has been Wildbolz's auto-urine test and its offspring the auto-serum test, have been devised.

1. *Auto-urine and auto-serum tests:* To repeat the statement that numerous tests have been devised for the diagnosis of activity in tuberculosis is to repeat a platitude. Few tests, however, have excited more interest than that of Wildbolz. Pirquet, Mantoux, Moro, Wolff-Eisner, Calmette—each thought that the results following his particular manner of applying tuberculin constituted a test for activity. But after extensive trial we know today that none of them does more than merely indicate infection with the tubercle bacillus. Wildbolz (232) in 1919, recognizing these facts, proposed a new test for activity on the hypothesis that persons with active tuberculosis eliminate "antibodies" or tuberculin-like substances in the urine. If the focus of tuberculosis is active, reasoned Wildbolz, products of bacillary disintegration or metabolism are formed, absorbed, and eliminated by the excretory organs —kidneys, skin, liver, intestines, etc. If active substances (antigens) such as these are in the urine, injection of the urine into hypersensitive skin should produce a reaction similar to that caused by tuberculin. In this test the situation is reversed in respect to the cutaneous reaction. Usually the cutaneous reaction is performed to determine whether the skin reacts, but in the Wildbolz scheme it is the urine

which is being tested as to its capacity to elicit specific reaction in skin which is known to be hypersensitive and which is, in such a circumstance, being used as an indicator of the specific substances in the urine.

As performed by Wildbolz the test consists of concentrating the urine which is later used as a tuberculin. Fresh morning urine, preferably about 150 cc., is collected, condensed to one-tenth of its original volume by evaporation in vacuo at about 55 C. It is then cooled and filtered through filter paper impregnated with 2 per cent phenol and 0.1 cc. of the filtrate is injected into the skin of the patient. Or, if he is not allergic to tuberculin, into the skin of a person who does have sensitiveness to tuberculin. The deltoid region is the site of choice and three injections are made fairly close together as follows:

Site No. 1	0.1 cc. concentrated urine.
Site No. 2	0.1 cc. tuberculin 1 to 1,000 dilution.
Site No. 3	0.1 cc. tuberculin 1 to 10,000 dilution.

Reading is done after 24 and 48 hours and later if indicated. The height of the reaction is generally reached on the second day. Both induration and redness usually characterize the reaction, but of these induration is of much more importance and constitutes a positive reaction in the absence of redness. When *B. Coli* are in the urine, violent redness but no induration follows the injection.

Criticism of the test promptly arose. May the reaction not be caused from the urinary salts? How much does normal variation in the concentration of voided urine modify the test? Will impaired renal function influence the results? What will be the effect of using urine from cases of renal tuberculosis and urine that possibly contains tubercle bacilli? Lanz (120), Imhof (89) and others anticipated and attempted to answer these questions. They applied the principle concerned in the auto-urine test by converting it, technique included, into an auto-serum test. They claimed that the auto-serum test substantiated entirely the reliability of the auto-urine test and that the reaction was specific and definite. Gibson and Carroll (69), Korns (102), Hayman (83), Bosch (21), Offenbacher (166) and many others have repeated both the auto-urine and auto-serum tests and have reported results that are conflicting. Better opinion would probably have it that, while the test may sometimes be of aid in diagnosis, it by no means records activity or latency in anything like a final degree, for in doubtful cases where one most desires a decisive reaction, it is usually equivocal, while, in full-blown tuberculosis, it is usually positive.

Non-specific Serological Reactions

There are likewise several of these tests, most of them based upon the stability or instability of the colloids of the blood and all have been promulgated as tests for "activity" in tuberculosis.

1. *Sedimentation of red blood cells:* When blood of normal persons is drawn in a solution of sodium citrate and allowed to stand, the red cells settle toward the bottom of the tube at a rate which is fairly uniform although somewhat more rapid in woman than in man. It is accelerated in certain physiological states (pregnancy and menstruation) and in various pathological conditions such as lues, acute infections with high fever, arthritis and tuberculosis. Observation of the phenomenon was made by Fahraeus (see (126), and first applied to tuberculosis by Westergren (21) who thought it a reliable means of diagnosis. Levinson (126), and Morriss (155), in this country have both brought the subject up to recent date and presented results of the test on the blood of tuberculous patients. It would seem from these results as from those of the rather large literature which has arisen on the subject that there may be and generally is a definite increase in the rate of sedimentation in active pulmonary tuberculosis and that the test helps in arriving at a better knowledge of the degree of progress. But as a test for diagnosis or activity, it fails. It does appear that when the test is applied serially at intervals of a few weeks or months to the same individual, it has considerable prognostic value—the more accelerated

rates carrying an unfavorable outlook.* The technique of the test has been modified considerably since first used by the earlier workers. As used by Morriss (155) and others, blood is drawn from an arm-vein in a syringe containing sufficient 3.8 per cent solution of sodium citrate to make a dilution of one to four, poured into wassermann tubes, shaken, and a portion drawn up into a calibrated 1 cc. pipette and this plugged by being put into a blind hole bored in a large rubber stopper. The pipette is then placed in a vertical position. Readings are made every 15 minutes for two hours, the reading expressing a definite percentage of the total volume of blood in the pipette. Normally, in healthy people, the two-hour reading should lie between 1 and 5 per cent in men and from 5 to 10 per cent in women. Elevation of the patient's temperature does not influence the rate of sedimentation. A large percentage of borderline cases of tuberculous individuals with symptoms but with few or equivocal signs, —precisely those patients in whom a diagnostic test would be most welcome, nearly all have a normal rate. In full-blown tuberculosis the rate is nearly always accelerated although a normal rate does not rule out activity. High rates do not contra-indicate specific therapy if clinically its use is warranted. However, if the rate becomes accelerated after institution of such treatment, the latter should be discontinued.

While in general it is agreed that lability of the serum may be at the bottom of the reaction, no definite, clear-cut explanation of the cause of the variation in the rate has been given. Various authors (see Morriss) have suggested, as explanation, changes (1) in the composition of the plasma such as an increase in the fibrinogen and a resulting agglutination, (2) in the volume and composition of the red blood cells and (3) in the surface tension, capillary attraction and differences in the electrical charges of the various blood elements.

2. *Daranyi's flocculation reaction:* In 1922 Daranyi (46) devised a test for alcoholic precipitation of the serum in tuberculosis, which he applied to 300 cases of active tuberculosis

Pinner (Arch.Path.and Lab.Med.,1926,II,513) revised his opinion and concluded that neither diagnostically nor prognostically is the test of any value.

and obtained consistently positive results that sera from healthy persons never gave. In the test 0.2 cc. of serum is added to 1.1 cc. of an alcohol-mixture prepared as follows: To one part of 96 per cent alcohol four parts of a 2 per cent solution of sodium chloride is added. The tube containing the mixture is placed in a water bath at 60° C. for 20 minutes, removed and read 1/2, 1, 2, 3 and 24 hours after removal. The reaction is characterized by the formation of a rather coarse, flocculant precipitate in the fluid; and, depending upon whether the reaction has appeared first at 1/2, 1, 2, 3 or 24 hours, it is designated as 4+(1/2 or 1 hour), 3+(2 hours), 2+(3 hours) or 1+(24 hours). These are the data given by Pinner (180) who used an additional tube containing alcohol in still greater dilution in an endeavor to refine the delicacy of the test. Readings should be made with the agglutinoscope. Pinner applied the test to 534 patients and pointed out that the test, being a measure of changes in the colloids of the serum, could hardly be expected to correspond accurately with a clinical classification of degree of activity. Of his active cases the test was negative in 10.9 per cent; of his "incipient active" cases, it was negative in 26.8 per cent, and in the far advanced cases, 7.6 per cent failed to react. In general the more rapid reactions occurred in those with more advanced disease.

In the experience of some authors, healthy persons do not give the reactions, while others have reported that a substantial percentage of the healthy react. More than 60 references are already available. The reaction is without help in diagnosis of *early* tuberculosis. Like several other tests for activity in tuberculosis, this one is generally positive in the cases in which there remains clinically no or but little doubt of the nature of the affection, and it is unable to select from the persons suspected of having early tuberculosis, those who really have it. The Daranyi test is so frequently present in active tuberculosis that its use has been suggested as a guide in therapy and general management of the case.

As Pinner has again pointed out, the explanation of the phenomenon is not entirely clear although several circumstances point to the increase in the amount of globulin in the serum as the most probable factor. It has been shown that the albumin-globulin ratio of the serum may become altered in tuberculosis, and chemists agree that globulin is more labile than the other colloids of the serum.

3. *Changes in chemistry of the blood:* The different substances in the blood may, and do, vary from the normal in different stages of tuberculosis. Changes in the water-content of the blood, variation in the serum-albumin-serum-globulin ratio, decrease in the total solids, increase in fibrinogen, fluctuation in the alkali reserve and in the enzymes have all attracted attention and have been well assayed by Wells (230). *But no changes that are of definite, specific, diagnostic or prognostic value have been found.*

Recently the demonstration of tuberculin-like substances in the serum (see under Wildbolz's reaction) and renewed interest in blood lipase have claimed considerable attention. Woolley (236) and others have shown that there is a distinct reduction in the serum lipase in advanced tuberculosis. Whether this is simply an index of "decreased functional activity which is associated with the wasting" remains undisclosed; it does not appear that the reduction bears any specific relation to tuberculosis.

Appraisal of Tests

Comment has been made in the description of each of these tests of its value, and it suffices to reiterate that not one of the tests mentioned is of itself diagnostic of tuberculosis or of activity in the disease. Certain of them have some worth in prognosis, but none is dependable for diagnosis. While the sedimentation rate of red blood cells does not aid in diagnosis, it and probably also the flocculation test may serve as useful guides to prognosis and possibly also to treatment. The auto-urine and auto-serum tests and others of their ilk have become routine in certain clinics and sanatoria because they are thought to be of aid in the general management of patients. Yet in a pinch where a diagnostic test is most needed, they, too, fail us.

PART V

SOME METHODS OF VALUE IN STUDIES OF TUBERCULOSIS EXPERIMENTALLY PRODUCED

CHAPTER XII

INOCULATION OF ANIMALS WITH THE TUBERCLE BACILLUS

Methods of approach to experimental problems in tuberculosis may be as widely variable as the disease is infinitely protean in its nature. Naturally, therefore, it would be out of the question to set down an account of methods that would be unqualified or complete. In general, experimental study falls into either a pathological or an immunological classification. These groups embrace anatomical, morphological, biological and bacteriological methods. But each of these may overlap and several of them may touch in a single experiment. In any discussion of such a subject it would, therefore, seem wiser to present a few of the broader aspects more or less as entities. And in the subjoined chapters such questions will be discussed as the inoculation of animals; the anatomical basis of the disease in the usual experimental animals, and the results, post-mortem, of the inoculation; recovery of the tubercle bacillus from animal tissues; description and dosage of types of tubercle bacilli in most common use; preparation of suspensions; various anatomical (injection, corrosion, etc.) and tissue culture methods; and statement of a few facts concerned in studies of immunity to the disease. There will also be mention of spontaneous diseases in experimental animals which resemble tuberculosis as well as consideration of histological methods of fixing, cutting and staining tissues.

Sporadic inoculation of animals and man in an effort to prove or disprove the contagion of consumption were reported from time to time long before the decisive work of Villemin (221) (222) in 1865 which proved unquestionably that tuberculous virus can be transmitted. Straus (210) has drawn attention to a number of early works on infection or contagion. Kortum (103), 1789, Hébréard (84), 1802, Salmade (191), 1805, inoculated pus from scrofula into children and animals. Lepelletier (124) in 1830 inoculated himself with similar material. None of these inoculations produced disease. Erdt (57), however, in 1834 inoculated horses with pus from scrofulous ulcers and claimed to have produced tuberculosis in them.

Animal inoculation is, of course, now very commonly done, and so many animals and so many types of inoculation have been used that it becomes a task to select from them the animal of choice and the technical procedure best adapted to the object in view at the time of a given inoculation. It shall therefore be the aim of this portion of the book to stress in some detail the animals of choice and the important procedures that may be called into service in the handling of animals or their tissues in circumstances in which experimental inoculation is concerned.

Animals of Experimentation

It is probably no exaggeration to say that nearly every type of animal, domestic or capable of any degree of domestication, has been used for inoculation in tuberculosis work. Rabbits, guinea pigs, dogs, cats, sheep, cows, calves, horses, goats, monkeys, apes, mice, rats, frogs, turtles, reptiles of various sorts, worms, fowl such as hens, pigeons and crows, have been used. Practically all mammals and fowl will succumb to tuberculosis if the proper dosage and the proper type of bacillus be administered, but long experimentation has taught that certain animals react more constantly and more typically than others and that, therefore, certain animals are better adapted for inoculation.

1. *Choice of animals:* Experience has abundantly demonstrated that for general work in tuberculosis the guinea pig and rabbit are animals of choice both because of their susceptibility to tuberculous virus, and their accessibility and relative cheapness. The guinea pig responds in typical fashion to inoculation with material containing bacilli of either human or bovine origin and is the animal of choice for general work. With the rabbit it is different. This animal reacts inconstantly to human bacilli. At times after inoculation with a human

strain, this animal develops a low-grade visceral, usually pulmonary, tuberculosis which may become chronic and which occasionally produces death. But the usual reaction of the rabbit to this type of bacillus is an abortive tuberculosis which heals, the animal remaining healthy indefinitely. If inoculation is done subcutaneously, signs of pulmonary or other visceral tuberculosis seldom develop. If intravenous inoculation is done, lesion develops in the various viscera, reaches a height at two or three weeks and later subsides, completely or in large part. But when the rabbit is inoculated with bovine bacilli the response is very different, for progressive, lethal tuberculosis ensues, regardless of the manner of inoculation. This fact gives the rabbit its unique and important position in the laboratory and makes it the animal of choice when a strain of tubercle bacilli is to be identified as to its human or its bovine origin. This subject has been considered in Chapter V.

Animals of any size may be used but in general rabbits should weigh from 1400 to 2500 grams and guinea pigs from 250 to 400 grams.

For work with avian tubercle bacilli, pigeons and hens are ordinarily used.

Inoculating Instruments

Requirements here are very simple, the chief requisite being that the syringe deliver the dose without external leak or leak between the piston and barrel. For ordinary inoculation, any type of accurately calibrated syringe may be used and the "Record" will be found to meet most needs very satisfactorily. Many people, however, use the "Luer" all-glass type with satisfactory results. But for intracutaneous inoculations the Becton, Dickinson & Co's, "Luer Tuberculin Fornier," or the "Sub Q" No. 2021, made by the Randall-Faichney Co., give particularly good results if, upon test, they have been found not to leak. Both the latter are graduated in hundredths of a cubic centimeter and are thus desirable when accurate measurement of small quantities of inoculum must be made.

Before use, any syringe designed for intracutaneous inoculation, where resistance is encountered and considerable pressure required, should be tested. The piston should fit tightly. A scheme for testing whether a syringe leaks has already been given (p. 208). It is seldom necessary to use a needle larger than a no. 20 gauge and perhaps never larger than a no. 18 gauge.

Where inoculation of tissue-emulsions is being done and a fresh inoculation apparatus is needed for each specimen, it is very convenient and economical of syringes to use volumetric or graduated pipettes connected by tubing to needles (p. 198).

1. *Care of the needles:* There is no good reason why needles properly cared for should not last for months of repeated use. The rule is, though, to see the shiny needle begin to rust after its second or third trip to the sterilizer. Needles will not rust, at least for a long time, if a few simple precautions are taken. These are: (1) Water should be drawn though the needle to wash out any coagulable matter before boiling takes place; (2) after boiling, the needle should be completely freed from water by drawing 95 per cent alcohol, and after that ether, through it several times; (3) the stylette, passed through either vaseline or thin oil, should be replaced in the needle where it should remain until the needle is again needed.

All inoculations should be performed over a pan. After the inoculation, scrupulous care should be given to sterilization of glassware, syringes, etc. The table should be washed with a disinfectant.

METHODS OF INOCULATION

The words *injection* and *inoculation* are used interchangeably in medical literature, but in a strict sense this should not be done. *Injection*, from the Latin *injicere*, means *to throw into* and is therefore a broad, inclusive term, embracing inoculation which, on the other hand, coming from the Latin *inoculare, to ingraft* (*or to furnish with eyes*) implies the introduction of viable material which, once introduced, may "bud" or grow. If we would use these two words in that sense, *injection* to denote introduction of inert matter and *inoculation* to denote introduction of viable matter into animals, the meaning of the terms would be more lucid. For years this has been routine in Krause's laboratory.

Inoculation of animals may be done in many ways. The most important are:

1. Subcutaneous and
2. Intravenous

Other methods are often used and for special purposes have advantages. They are:

3. Intraperitoneal
4. Intracutaneous
5. Percutaneous
6. Orbital
7. Intrapleural
8. Intraspinous
9. Inhalation or insufflation
10. Ingestion

The details of these several methods follow.

1. *Subcutaneous:* This simple and commonly used method of inoculation may be done in approximately the same manner in both guinea pigs and rabbits, but is employed much more extensively in the former animal. It is better than intraperitoneal inoculation for routine work. The inoculation may be made under the skin in any portion of the body, but certain regions, namely, the right and left groins, have been found to have advantages. In these areas the relatively thin, loose skin facilitates inoculation. Here, too, the superficial inguinal lymph nodes are situated and these invariably become enlarged and diseased after introduction of tubercle bacilli into the inguinal space. For the sake of convenience it is well to inoculate regularly in either one groin or the other. In our laboratory, guinea pig-inoculation of material for diagnosis is routinely done in the right groin and is entered in the records, "S. Q. R. G."

The procedure is carried out as follows: The assistant holds the animal flat upon a pan, back down, caudal end toward the operator, with the hind legs well spread. The hair and skin in the right groin is wet with a pledget of cotton soaked in 2 per cent carbolic acid or some other disinfectant. With the thumb and forefinger of the left hand, the skin of the region is lifted up slightly and the syringe needle, which is held in the other hand, is passed through the skin a little lateral and caudal to the mammary gland. The properly situated needle can be easily palpated through the skin and is freely movable in a lateral plane. Occasionally one passes the needle into the muscle of the thigh or belly, but this is easily determined, for in such circumstances the needle is not movable from side to side and its tip is not palpable. After the inoculation is made, the needle is withdrawn and the area rubbed again with the carbolic sponge. The position of the animal and syringe as well as the landmarks are shown in figure 12. The procedure is much the same in rabbits except that much more wetting is required and inoculation is performed relatively somewhat more medially than in the guinea pig. The technique of holding the animal is given on page 212. If it is necessary to inoculate several different substances at a given time, the use of pipettes instead of syringes, as has just been indicated, is a very convenient manœuver. A piece of rubber tubing $2\frac{1}{2}$ inches long is slipped over the syringe-end of the needle. This is then boiled and attached to a sterile volumetric pipette. Such a combination is used for the inoculation of one specimen of inoculum and then is set aside. A second pipette and needle are used for the next inoculation and so on. A small rubber tube about the length of the pipette is used to effect a connection between the latter and the mouth. A "suck tube" of this length allows free play of the pipette and permits one to read the calibration while governing the pressure with the mouth. The tube remains in the mouth throughout the inoculations and is attached to each new pipette in its turn. The needle is held in the right hand and is passed into the skin as in an ordinary subcutaneous inoculation; the pipette is allowed to rest on the lower right arm and pressure is made with the mouth (figure 13). The pipettes may be sterilized by standing in a jar of cleaning solution (p. 3) after completion of the inoculation. The needles and rubber may be washed out with water and boiled.

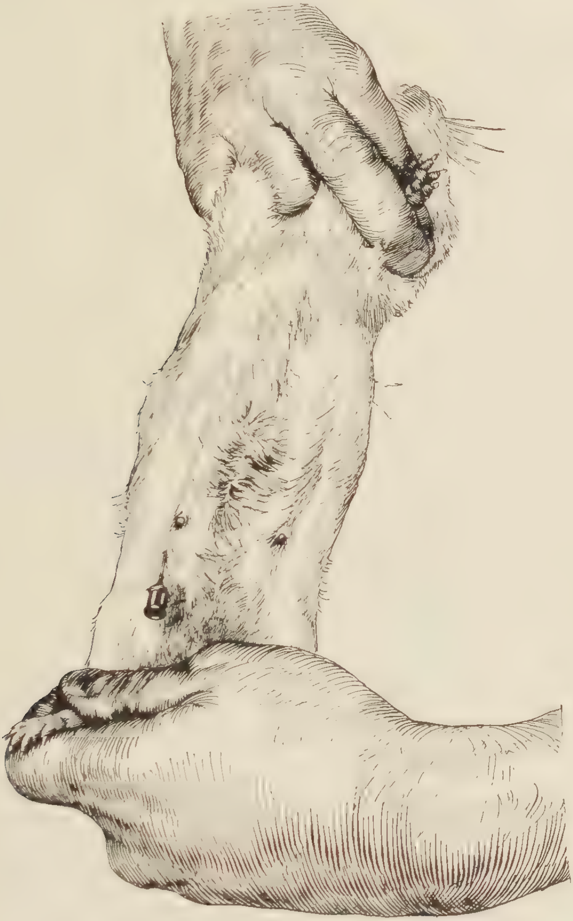

Figure 12. Line Drawing of Technique of Holding Guinea Pig for Subcutaneous Inoculation

Description is given in the text (p. 212).

In any type of inoculation it is always very helpful to steady the syringe against sudden movements of the animal by placing the fingers of one hand on both the syringe and the

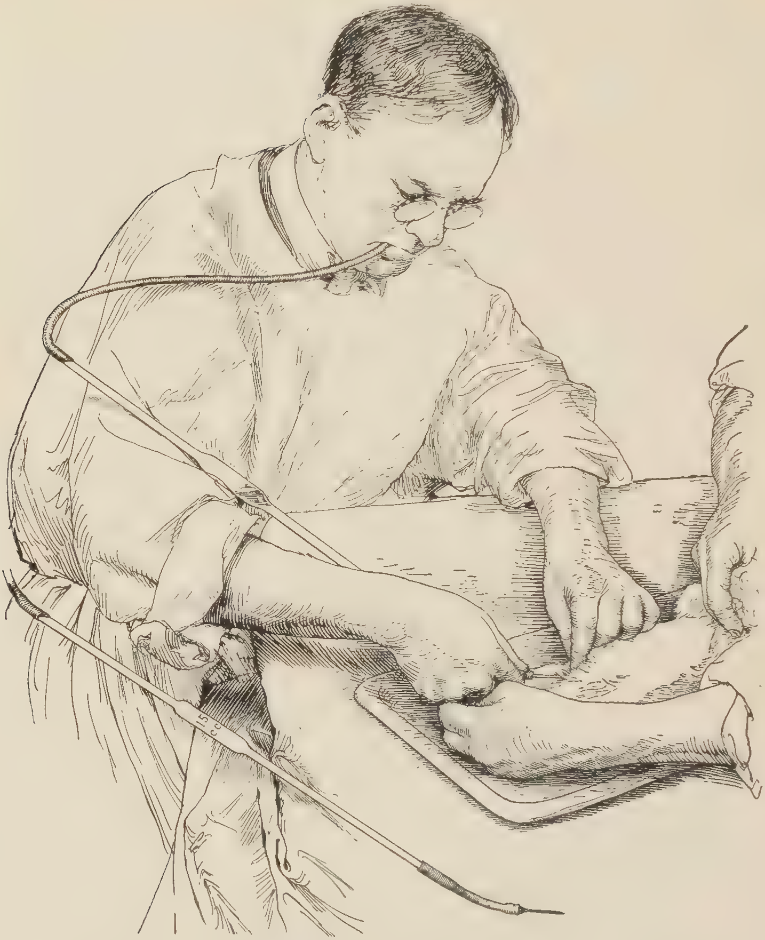

Figure 13. Drawing Indicating Another Mode of Subcutaneous Inoculation

The use of pipette and "suck-tube" is very convenient and saving of syringes when several different specimens require inoculation at a time.

animal. For instance, one is doing a subcutaneous inoculation in a guinea pig: With the syringe in the right hand, the skin of the animal is held with the thumb and second finger of the left hand while the first finger of this hand is held against the butt end of the syringe. Such a procedure tends to fix the animal, the hand and the syringe as a more or less rigid system, all of which will move together in the event that one member of the system (the animal) moves.

2. *Intravenous:* In the *rabbit*, which has numerous large veins superficially located, this mode of inoculation may be executed easily and quickly. The ear is selected and the hair along the lateral, marginal vein shaved, the necessity of shaving depending upon how well the vein can be seen. The peripheral rather than the central vein should be used because the latter is much more sensitive to pain. The animal is held firmly by the assistant who sits on the opposite side of a narrow table from the operator and close to it. He gently draws the rabbit backward until it is sitting in a hunched position with its tail pressed against the assistant's body and its head directed toward the operator. He then places his arms and hands on either side of the rabbit and a little dorsalward, and, with the thumb and forefinger, clamps the marginal vein at the base of the ear designed for inoculation. The procedure is illustrated in figure 14. Frequently this pressure does not cause the vein to dilate suffi-

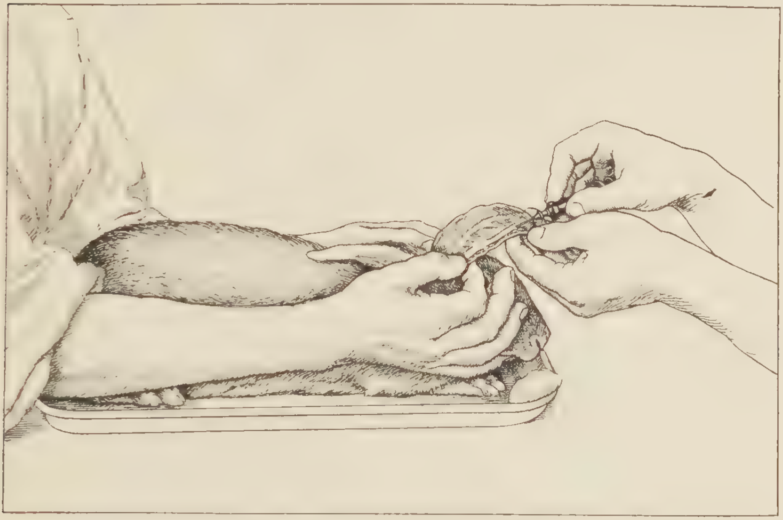

Figure 14. Drawing Which Illustrates Method of Holding Rabbit for Intravenous Inoculation

ciently. When this is the case, the ear may be slapped or taken between the two hands of the operator and rubbed vigorously, twisted and crumpled several times. Such procedures nearly always give the desired distention to the vein. Some workers recommend the application of an irritant such as turpentine or xylol to the ear. These substances will always produce dilation, but they are not necessary and, by their irritating properties may injure the ear, or complicate the results. After dilatation, the needle (20 to 23 gauge), directed centralward, is inserted into the distended vein on which pressure is now released by removal of the assistant's thumb and finger, and the inoculation is made by gentle pressure on the plunger. Another and perhaps more satisfactory scheme for holding the animal is by employment of a box with a sliding lid so notched in the end that, with the animal inside, it closes about the latter's neck and holds the head in position. This furnishes a better guarantee against the animal's moving than the holding method. Figure 14 illustrates one method of intravenous inoculation.

The manœuver in the *guinea pig* is attended with considerably more difficulty because this animal has no superficial veins of caliber sufficient for passage of the needle, and surgical procedure is necessary to expose suitable veins. The internal jugular is the vein of choice. The vein on the right side is more easily entered when the operator holds the syringe in his right hand. The animal is tied down rigidly upon its back on a convenient board or frame with structures at the four corners for holding the leg ties. A useful frame is illustrated in figure 15. The noose surrounds the hind leg above the ankle but on the front leg it must fit tightly above the knee joint. A small piece of wood, such as a strip of one inch, half-round moulding about three inches long placed under the back of the neck raises the field of operation advantageously. The head of the animal, directed toward the operator, should be held immovable by the assistant in the event anaesthesia is not administered.[19] The neck is shaved and an incision about 3/4

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[19] It is well to use ether unless the experiment contraindicates it. But it is surprising to see how, without anaesthesia, the animal lies placidly in no apparent distress while this inoculation is being performed.

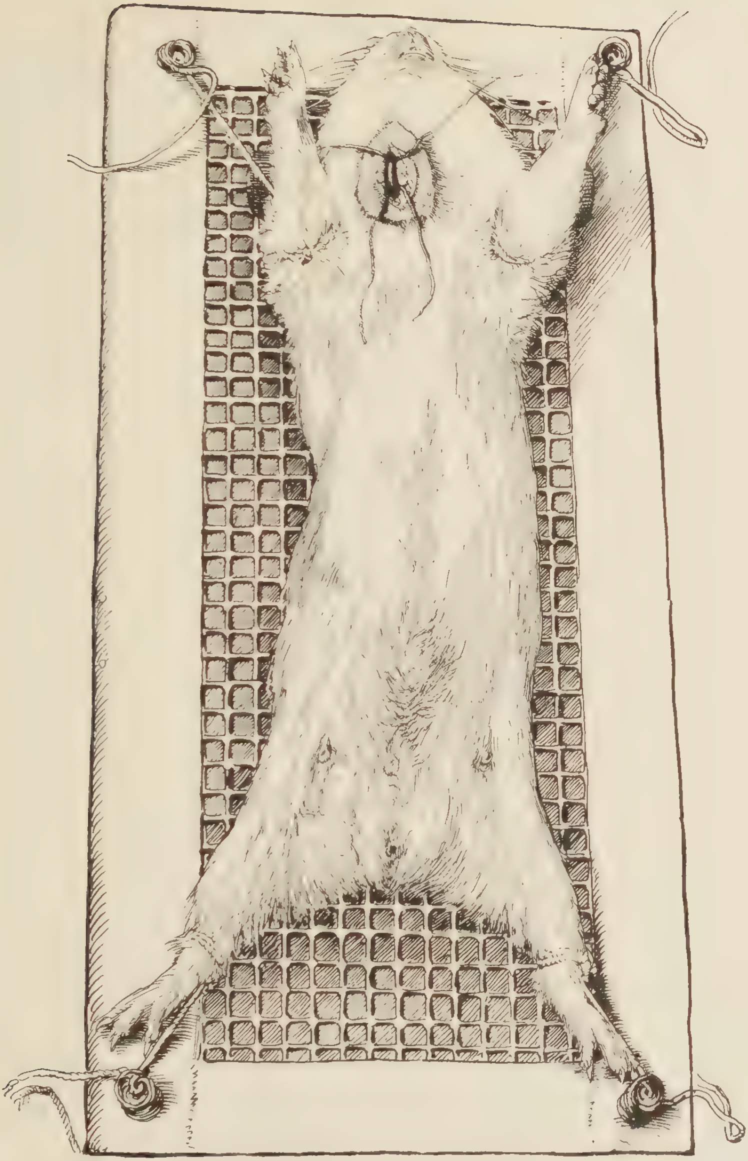

Figure 15. LINE DRAWING SHOWING PROCEDURE OF INTRAVENOUS INOCULATION IN GUINEA PIGS

The animal is tied down to the frame (which is all metal and can be readily sterilized) and has a small block of wood under the neck to raise the field of operation. The internal jugular vein is exposed and two ligatures placed under it.

of an inch long is made in a line which passes from the cephalic end of the sternum to the angle of the jaw. By blunt dissection the vein is exposed and two silk ligatures are laid about it, one-fourth of an inch apart. Curved forceps are passed carefully under the vessel for placing the ligatures: a small artery clamp (figure 16) is placed just caudalward to the proximal ligature, and the vessel becomes considerably distended. A second clamp is placed cephalward to the distal ligature. The needle, of 25 to 27 gauge and one-half inch in length is introduced

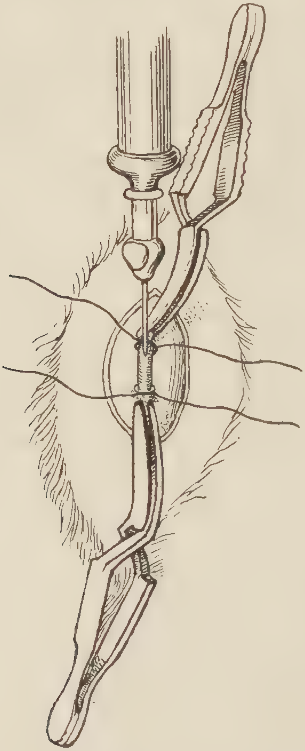

Figure 16. Drawing Illustrating Intravenous Inoculation in Guinea Pigs. A Companion to Preceding Drawing

The ligatures have been lightly tied, the vessel clamped with small artery forceps and the needle has been introduced. Details of the inoculation are described in the text.

into this distended, sausage-shaped section of the vein in a plane nearly parallel to the vessel and directed toward the body; the proximal clamp is released, and the inoculation slowly made. The assistant then ties the proximal ligature around the needle and tightens it as the needle is withdrawn: the other ligature is tied, the clamp removed, the edges of the incision are brought together with a stitch and covered with celloidin. The animal is returned to its cage, where it almost invariably will promptly begin to eat. Some workers make the inoculation without the use of ligatures and depend upon pressure on the vein to prevent subsequent bleeding.

The instruments, needles, etc., should be sterilized, and the ligating silk washed with a disinfectant before the incision is made, but the area almost of necessity becomes contaminated to some extent. So long, however, as contamination is not gross, the animal suffers no ill effects.

Intracardiac inoculation is occasionally resorted to. It is, however, an uncertain quantity as one cannot always be sure whether the needle has entered the right or the left heart. Distribution of early lesions might very well differ in animals inoculated indiscriminately into the pulmonary or systemic circulation. A needle, unattached to a syringe, is introduced by a quick stab along the left sternal border at about the junction of the caudal with the middle third of the sternum and vertical to this structure. When blood flows freely from the butt end of the needle, a syringe, already containing a measured dose, is attached, and the inoculation made. The needle is removed with a quick jerk. The color of the blood will indicate fairly well whether the needle is in the left or right heart.

3. *Intraperitoneal:* Use of this mode of inoculation is limited to pure cultures or tissues free from other microörganisms than tubercle bacilli, because of the possibility of fatal peritonitis, which may follow the inoculation of sputum, urine, faeces, or other matter containing other bacteria. There is also another valid objection to frequent use of intraperitoneal inoculation. The peritoneal cavity has a remarkable capcity for ridding itself rapidly of foreign matter, and, when only a few tubercle bacilli are introduced by this route, the chance of

infection is less than if inoculation were done in the groin. This is not to say by any means that infection cannot or does not occur from intraperitoneal inoculation. It can and does. But the possibility of "missed infection" by this route is greater than by the other, although once infection is established in the peritoneum, it spreads rapidly over the body.

The technical procedure is exceedingly simple. The assistant holds the animal, belly up, on a pan with head away from the operator. The belly wall is wet with carbolic acid in or near the midline. Then the skin is lifted up and the needle is introduced in a horizontal rather than vertical plane through the skin and belly wall just where the skin is pulled away from the underlying structures. After it is introduced the needle is directed somewhat dorsalward, the inoculation made, the needle removed and the area made wet again.

4. *Intracutaneous:* This mode of inoculation is frequently used for the administration of tuberculin both clinically and experimentally. In experimental work such a method finds a place where it is desirable for one reason or another to study local tuberculous infection or where one desires to trace bacillary dissemination from local foci to remote and inner portions of the body. It also serves a useful purpose for comparative study with other types of tuberculous infection.

The technique is simple (Mantoux (145)). An area on the animal is shaved preferably about half way between the axillary and inguinal regions. The assistant holds the animal taut, flat on a pan and parallel with the long diameter of the table with the shaved side upward. The needle should be about the same size as that used for intravenous work, namely, 23 to 27 gauge and one-half inch in length. It is directed caudalward.[20] The very tip of the needle is buried in the skin which is held between the thumb and first finger (figure 17). The syringe is given a one-half turn just before inoculation. Syringe and needle are given a further slight twist in the same direction as the first turn and then quickly withdrawn. No oozing occurs

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[20] This is done because, when the path of the needle crosses the tract of the hair-follicles, the former is more quickly obliterated after needle withdrawal than when the two paths correspond.

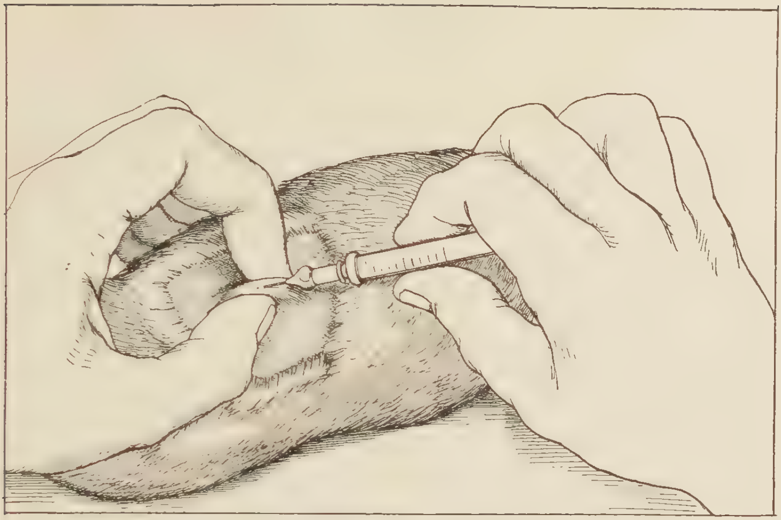


Fig. 17

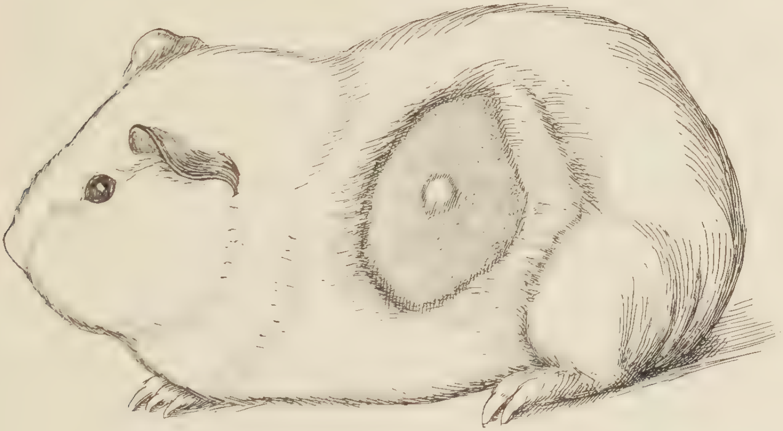


Fig. 18

Figures 17 and 18. DRAWINGS OF TWO STAGES IN INTRACUTANEOUS INOCULATION

In figure 17, the right hand of the operator is purposely placed further along the barrel of the syringe from the needle than it should be, to prevent crowding in the drawing. Figure 18 illustrates the area at the cessation of inoculation.

if the removal of the needle is done with a little jerk after the twist. Passage of the needle too far into the skin is likely to produce subcutaneous inoculation. If the needle is *in* the skin, a little, tense, white bleb develops as the inoculum is forced in (figure 18): if the needle has passed *through* the skin no such bleb occurs and only a slight, diffuse, transient swelling is seen. Only slight pressure is required to make the subcutaneous but perceptibly more is necessary for the intracutaneous inoculation.

The side is the optimal site for inoculation. The skin on the belly of the animal is very much thinner than that elsewhere over the body, and intracutaneous inoculation in this situation may frequently become subcutaneous because of the ease with which the needle may pass through the skin. On the back, on the other hand, the skin is so thick as to make the introduction of the needle, if of small gauge, and the inoculation of material more difficult than the skin of the side. The thickness or thinness of skin, however, are not contraindications to inoculation if such sites are desirable, for infection occurs in typical manner in the skin in any situation. However, it is interesting that the local reaction is of less intensity in the ventral than in the dorsal skin of the guinea pig. This is true of reaction to tubercle bacilli and to tuberculin.

Considerable pressure is required for this type of inoculation, and selection of a nonleaking syringe is important. The syringe may be tested very simply. A needle is firmly attached to the syringe to be tested (which has been filled half full of water); the needle is then stuck into the depths of a rubber stopper and pressure exerted about equal to that required for making an intracutaneous inoculation. If there is seepage between the piston and barrel of the syringe, the syringe is inadequate for intracutaneous inoculation although it may perfectly well be used for other modes of inoculation where pressure requirements are not so important.

Shaving the skin with a razor produces practically no trauma or irritation and is quite satisfactory, but a depilatory may be used. Lewis and Aronson (127) have recommended a depilatory made as follows as being non-irritating.

> Mix barium sulphide with a saturated solution of sodium sulphite and add to this white of egg and water until the mixture assumes a paste-like consistence. The hair is cut with the scissors, the dipilatory applied for one or two minutes and then scraped off, after which the part is washed with water.

5. *Percutaneous:* The inoculum may be rubbed into the skin and infection established in this manner. The area of the rubbing should be in a situation that cannot be licked by the animal. The skin of the back of the neck is usually recommended.

6. *Orbital:* The eye may be thought of as a logical or "natural" site of inoculation in so far as it is possible to effect an inoculation in this location without trauma to tissue(for instance, by instilling bacilli into the conjunctiva). Inoculation of the cornea or anterior chamber affords one an excellent opportunity to observe the slow, leisurely development of macroscopic tubercle. Generalized tuberculosis follows orbital infection in animals, but, except in conjunctival infection, the development of it is somewhat delayed over that of most other methods of inoculation. If inoculation be corneal, intraorbital, or post-orbital, a drop of some local anaesthetic should first be placed in the eye.

a. *Conjunctival:* It is simple and easy to infect the conjunctiva by merely instilling a drop of bacillary suspension into the eye while the lids are held open. The eye-lids should be drawn slightly from the bulb in closing so that none of the drop overflows (Calmette (31)).

b. *Corneal:* If corneal inoculation be desired, it can be done by simply inserting a needle containing tubercle bacilli into the corneal layers, introducing only the slightest possible amount of the suspension and withdrawing the needle. Equally satisfactory results may be secured by scarifying the cornea with a needle or scalpel and applying tubercle bacilli to the area with an ordinary transfer loop or spade.

c. *Intraorbital:* Intraorbital inoculation requires a certain degree of finesse. The eye is steadied through gentle pressure by the assistant's finger upon the lateral aspect and is further immobilized by a small mousetooth forceps which holds the conjunctiva just lateral to the cornea. The cornea is penetrated in an oblique direction by a small needle and one or two drops of aqueous humor allowed to escape. The syringe is then applied and a small amount of bacillary material is inoculated. A bulk of the inoculum greater than the amount of aqueous humor removed should not be introduced. The needle is quickly removed and the eye washed with sterile physiological saline.

d. *Postorbital:* This is an important mode of introducing foreign matter, especially tuberculin, into animals. Absorption occurs with great rapidity, and numerous studies in hypersensitiveness and anaphylaxis have been carried out by utilizing this method. It has been used also in inducing tuberculous meningitis experimentally in animals. The eye is held by gentle pressure at the lateral commissure and the needle worked into the orbital cavity by a rotary motion. The needle is allowed to make contact with the eye at its lowermost surface and is passed sidewise and backward until the tip lies behind the eyeball and the shaft between the eyeball and the nasal side of the orbit. By movement backward and upward the needle is brought into the optic foramen for inoculation. The animal suffers no ill effects, but the technique is rather difficult.

7. *Intrapleural:* Pleural tuberculosis and experimental pleural effusions are set up by this type of inoculation. In doing it the thorax is shaved on one side, sponged with 70 per cent alcohol and the needle is introduced through one of the interspaces. The needle should enter obliquely so as to have less opportunity of puncturing the visceral pleura and it should be passed in only as short a distance as is consistent with reaching the thoracic cavity. The needle may remain unattached to the syringe and may contain a stylette until after the cavity is reached, when the stylette is removed and the syringe attached. No resistance at all is encountered in doing the inoculation.

8. *Intraspinous:* Austrian (6) has suggested the use of lumbar puncture as a highly satisfactory means of inducing meningitis in rabbits and of testing whether material (such as spinal fluid) from patients contains tubercle bacilli. Lesion develops promptly after this type of inoculation. The skin in the lumbar region is shaved and cleansed with iodine; light ether anaesthesia is given, the animal is put on its belly, and the needle passed between the spines of two lower vertebrae. Either one's tactile sense or the contraction of the rabbit's hind legs or tail will indicate that the needle has entered the canal or touched the cord. The syringe is attached to the needle and inoculation is made slowly. An inoculum of one to two cc. is readily tolerated by the rabbit, the larger dose being given usually to larger animals. Dyspnoea, periodic respirations, a short period of apnoea, or clonic spasms and rigidity of the neck are apt to be present for a few seconds, but are of no moment.

Soper and Dworski (207) employed the so called *occipito-atlantoid* method, in reality a modified postorbital method, in which the rabbit, under complete anaesthesia, is laid on its side and the needle, 26 gauge and three-fourths to one inch in length, passed into the orbit medial to the eye-ball, is gently carried medially and backward into the optic foramen and through it. A small amount of fluid is withdrawn and an equal quantity of suspension inoculated. Meningeal tuberculosis is readily induced.

9. *Inhalation:* For certain purposes, infection by inhahalation may be resorted to with profit. Details of such a procedure, however, had best be worked out to meet the specific requirements of the case in hand. Baldwin and Gardner (13) placed a guinea pig in a rubber cone with an aperture just large enough to snugly accommodate the animal's nose and then sprayed bacilli in moist suspension or in dry powder at the animal. But even with the best technique, it is improbable that a *pure inhalation* experiment can be devised (Krause (107)). When tubercle bacilli are blown or sprayed at guinea pigs' noses, some of them very likely stick to the pharynx and are swallowed or are washed by mouth-currents into

the oesophagus and stomach. Even in tracheal insufflation a certain number of bacilli must be swept up the trachea and thus possibly swallowed or carried to the tonsils and cervical nodes. So, while inhalation can be done satisfactorily and advisedly, it cannot be used to prove routes of infection or portals of entry.

Intratracheal inoculation may be easily done. With the animal supine, the neck is shaved over the trachea, the skin washed with a disinfectant, the trachea held closely under the skin with the fingers which grasp it and the needle passed through the skin and tracheal wall. This procedure becomes somewhat easier if the trachea is exposed by an incision in the skin. By this means there is less likelihood of pushing the needle completely through the trachea into the structures of the neck. When the suspension gets into the trachea, râles are nearly always audible, and the animal will frequently sniff or cough out some of the inoculum. To prevent a spread of this a piece of cotton should be held over the nose immediately after the needle has been withdrawn (8).

10. *Ingestion:* Infection by the digestive tract has been accomplished by the stomach tube, by feeding bacilli in milk or boli of food or gelatine capsules, by inoculation into the rectum. Details will vary with the needs of the case in point.

Inoculation of Fowl

When fowl (hens, pigeons) are employed for inoculation, either the intraperitoneal or the intravenous mode is used. In the former, a few feathers are plucked in an area about half way between the sternum and the cloaca. The skin in this region is then washed with carbolic acid and the inoculation made intraperitoneally. If intravenous injection be desired, the bird is held on its back, a vein in the third joint of the wing on the medial surface is found, the area washed and the needle slipped into the vein. Fowl may also be very readily infected by feeding.

Technique of Holding Animals

Animals may be held in position for inoculation in a number of ways, various types of inoculation naturally indicating various positions. Since subcutaneous inoculation is by far the commonest method and the one which we are required most frequently to perform, attention shall be paid to holding the animal for this particular type of inoculation. Slight variations from this method make it applicable to the special needs of several types of inoculation. Holding a guinea pig for inoculation is at once simple and difficult. The animal is an excitable creature and moves by jerks and without warning. The first requisite is that the animal be held belly up, flat upon a pan on the table. The assistant holds the hind legs with his left hand, the animal's left foot between his second and third fingers and the right foot between his thumb and forefinger, which are in extension in order to spread the legs. With his right hand, the assistant holds the animal's head and fore feet, his thumb on the occiput, his forefinger on the left mandible, with the animal's left foot held firmly between this finger and the middle finger. The ring finger, flexed on itself, presses against the right mandible and the little finger holds the right paw firmly against the palm. Thus no pressure is made upon the trachea; the head and all four feet are held rigidly and the animal may be easily stretched at the discretion of the assistant into a position from which it cannot move. Figure 19 illustrates the method.

Methods for holding the rabbit have already been given in the section on intravenous inoculation.

Chapter XIII

INDUCED TUBERCULOSIS IN ANIMALS

Anatomy of the Lymphatic System of the Guinea Pig and Rabbit

Before considering the results of inoculation, a word should be said about the anatomical basis of tuberculous infection in the animals ordinarily used in experiment. Particularly is some knowledge of the lymphatic system in the two commonly used animals of experimentation requisite to an understanding of the spread and development of tuberculosis after the various modes of inoculation. This is so because it can probably be laid down as a truism that tuberculosis has its beginning within "the domain of the lymphatics" and is disseminated in large measure by this system; and this irrespective of the mode of entrance of the infecting agent into the body. Brief comment is therefore pertinent upon a few of the more important relationships of this system in these animals, respecting both the relations to the body in general and to certain organs in particular. This will be given in barest outline, and the reader is referred to the paper of Hashiba (82) who has carefully worked out the details of the lymphatic anatomy of the guinea pig and to that of Krause (110) who has gone into the important features of the anatomy and physiology of the lymphatic system of this animal in so far as they relate to experimental tuberculosis. The latter author (112) has later compared some of the significant features of the lymphatic system in the two animals under consideration.

1. *Guinea pig:* The guinea pig possesses a rich and abundant supply of lymphatics, both superficial and deep. Several features of these will be pointed out in the order of the spread of tuberculosis from a superficial point of inoculation.

a. *Superficial inguinal nodes:* These small nodes, five or six in number, lying just beneath the skin of the groin in a pad of fat, range in size from a diameter of 2 to 5 or 6 mm. In the normal state they are barely palpable and, as may be seen in the accompanying figure (figure 19), they are intercalated along small lymphatic channels which continue centralward to the deep inguinal and iliac nodes. The lymphatic connections *between* these superficial nodes may not always

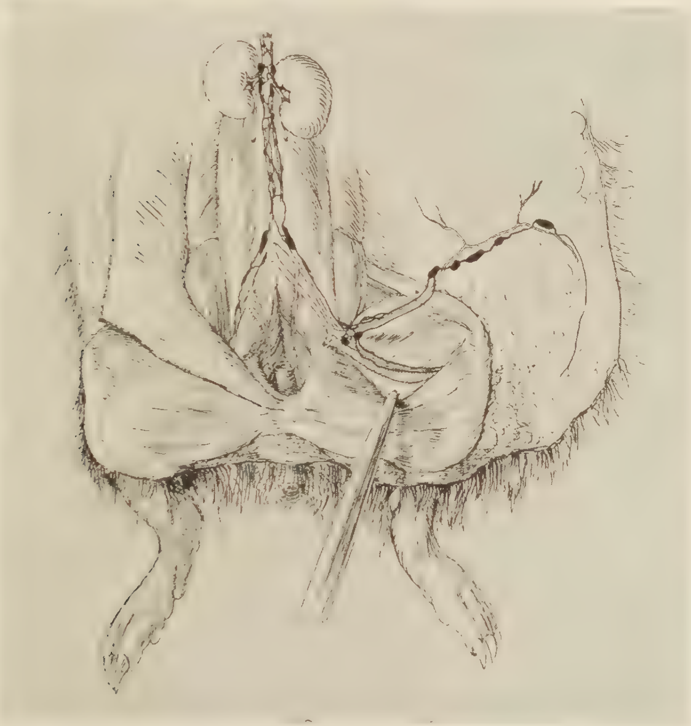


Figure 19. DRAWING SHOWING INJECTED LYMPHATICS AND LYMPH NODES OF GUINEA PIG

Injection was made in the pad of the foot and nodes and lymphatics dissected out. The situation of the superficial inguinal nodes in the groin (the usual site of inoculation) and their relation to the more central groups of nodes is well represented.

be perfect, however, or bacilli may pass through nodes without initiating gross lesion because, after injection in the groin, not infrequently the first one or two nodes in the chain, as well as the deep inguinals and iliacs on the side of inoculation, may bear gross evidences of tuberculosis, while two or three of the deeper superficial nodes remain normal to unaided vision. Cutaneous lymphatics from the scrotum, abdomen and thigh drain into this chain of nodes.

b. *Deep inguinal nodes:* These two small nodes lie at the inguinal ring and receive lymphatics from the superficial inguinal nodes, the popliteal node and probably the entire leg and send one lymphatic through the inguinal canal to the common iliac node.

c. *Common iliac nodes:* Through the lymphatics that pass from the superficial and deep inguinals, these nodes become receiving stations for lymphatics from the pelvis and entire hind leg and the caudal half of the skin of the body. They are lenticular in shape, about 6 or 7 mm. in length and not more than 2 mm. in width and lie usually lateral to the great vessels at their bifurcation. One node is usually present on each side, but there is frequently a small one in the bifurcation itself.

d. *Paraortic nodes:* Lymphatics leave the common iliac nodes and pass cephalward along the abdominal aorta about which they break up to form a fine network that extends beyond the kidneys to the cisterna chyli. Along this plexus there are numerous very small nodes which are just at the point of vision when normal and uninfected but which are easily seen when the animal has been recently infected in the groin with tubercle bacilli. The most anterior of these nodes lies just beneath the kidneys and is larger than the others of the network.

e. *Cisterna and thoracic duct:* The plexus as it continues anteriorly merges into the cisterna chyli which, passing through the diaphragm, becomes continuous with the thoracic duct. This latter structure virtually drains everything caudal to the diaphragm and conveys its contents to the venous blood just before it enters the heart.

f. *Lymphatics of the lungs: tracheobronchial nodes:* Whatever foreign matter may be brought lymphatically to the blood stream is carried directly from the heart to the lungs. If this gets from the capillaries into the tissue spaces of this organ, it is again in the "domain of the lymphatics," for, communicating with these spaces, are large, sinuous lymphatics that course in the walls of the arteries, bronchi and veins. Associated with these lymphatic vessels there are occasional nodes or masses of lymphoid tissue. The lymphatics of the pleura and the lungs drain to the tracheobronchial lymph nodes at the hilum (Miller (150)). These nodes are relatively large, the average one measuring 8×5×2 or 3 mm. There are usually four or five of them and they lie in or near the tracheobronchial angles and in the angle made by the bifurcation. They receive lymph from the lungs and pleura, the heart and the lower trachea and send efferents to the retromanubrial and axillary nodes and through these to the plexus on the innominate vein and thence to the lymph and blood stream. *They have no connection with the cervical lymph nodes.* With the large, open lymphatics in the lung and great paucity of intrapulmonary lymphoid tissue, conditions are such that the flow of lymph through the organ to the tracheobronchial lymph nodes must be rapid and relatively unimpeded.

g. *Lymphatics of the liver:* The liver, like the lung, has but little lymphoid tissue and, again, like the lung, has a large node at the point of emergence of its lymphatics; namely, the retropancreatic node (Hashiba). Drainage from this node is into the plexus about the abdominal aorta.

h. *Lymphatics of the spleen*: This organ in the guinea pig is conspicuous (1) for the large size of the malpighian bodies and thus for many nodules of lymphoid tissue and (2) for the inconspicuousness of its node of drainage. This may explain, at least in part, the predilection of this organ for tuberculous infection, as compared with the rabbit's spleen which, smooth and without conspicuous malpighian bodies, relatively escapes infection. The anatomy of this organ and the kidney in these two animals, however, must be worked out before anatomical differences can be credited solely with being the cause of the obvious differences in infection with the tubercle bacillus.

2. *Rabbit*: Much less is known about the lymphatics of the rabbit, but it appears that the lymph nodes tend to be less conspicuous throughout the animal except in the lungs, where conditions differ materially from those in the guinea pig. In the rabbit's lung the lymphatics are small and inconspicuous and, more important, there is a great deal of intrapulmonary lymphoid tissue present. Intercalated at close intervals along the lymphatics, and especially at dividing points of bronchi and vessels, there lie relatively large masses of this tissue which act as filters that may hold tubercle bacilli which are in transit toward the hilum. Correlated with this, too, is the fact that the tracheobronchial lymph nodes are inconspicuous bodies usually so small that in about one-third of the animals they are not found in ordinary dissection (Willis (234)). Lymphatic drainage through the lungs of this animal must be exceedingly slow and halting, as compared to that in the lungs of the guinea pig.

Results of the Inoculation of the Guinea Pig

1. *During life of animal:* a. *General:* 1. *Atony:* As a rule, the only general sign of progressive tuberculous infection in the guinea pig consists of the development of a body laxness and loss of tone, which when present, appears three, four or five weeks after infection, but it is not always present. The animal loses its normal springiness and muscular alertness and, upon being picked up, hangs flabbily with but slight struggle to free itself. However, such atonic states ensue in the course of several epidemic and endemic diseases to which laboratory animals fall heir, and it is of no real value in determining whether the animal is infected with tuberculosis.

2. *Fever:* Guinea pigs and rabbits have fever during the course of their tuberculosis, but it is inconstant, and very little reliance can be placed upon its detection because of the very wide fluctuation in the temperature of these animals in the normal state. Temperature in normal guinea pigs and rabbits is notoriously unstable. Even the excitement incident

to picking up the animal or to taking its temperature may cause a rapid rise.

3. *Loss of weight:* In general, the same inconstancy characterizes the weight of normal animals, especially guinea pigs, and suffices to prevent reliance on weight as a criterion by which progress of the disease may be determined. Late in the course of the disease the animal loses considerable weight, but frequently autopsy upon an animal (guinea pig) dead of tuberculosis will reveal a good deal of fat. When young, growing guinea pigs are infected, they frequently remain stationary in their weight while their bony framework is growing and they give an impression of great emaciation.

4. *Sensitiveness to tuberculin:* When an animal reacts to tubercle bacilli to form anatomic tubercle, it becomes sensitive to and will react to tuberculin. This is administered in doses of 0.1 cc. of a 3 per cent solution of Old Tuberculin or 0.2 cc. of a solution made up of equal parts of Water Extract and physiological saline (page 262). Reaction develops within 24 hours. Test with tuberculin to determine if the animal is infected is rarely necessary.

b. *Local:* 1. *Lymph-nodal reaction after inoculation of pure cultures:* After a subcutaneous inoculation of a suspension of tubercle bacilli in the groin of the normal guinea pig, uniform and constant results ensue. For a week or ten days the site of inoculation remains in an apparently normal state, but after this time there occurs a slow evolution of the first signs of infection, namely, enlargement and hardness of the superficial inguinal lymph nodes. This begins as a small, hard, discrete, shotty tumefaction which slowly but progressively enlarges to become the size of the end of one's finger, and conglomerate, as other nodes in the chain become diseased. The area frequently ulcerates several weeks after inoculation.

2. *Non-tuberculous bubo-formation after inoculation of contaminated material:* The inoculation of contaminated material subcutaneously gives a different type of reaction from that produced by inoculation of pure cultures. Although animals, especially guinea pigs, are highly resistant to the bacteria commonly found as contaminators in the material which we

are often called upon to inoculate, the introduction of such microörganisms produces certain demonstrable changes in the animal. The chief of these is the development of bubo at the site within two or three days of the inoculation. This swelling reaches its height at five to seven days and nearly always ulcerates, the swelling at times reaching the size of an English walnut and completely filling the groin. Directly upon ulceration the reaction subsides and by the eight or tenth day it has almost completely disappeared. At about this time or a few days later, a secondary enlargement begins to develop in the groin if tubercle bacilli were present in the inoculum. This tumefaction is characterized by firm, shotty hardness of the superficial inguinal nodes that occurs after infection with cultures of tubercle bacilli, and it occurs in typical manner regardless of whether early swelling or ulceration had been present. This is the true reaction to the tubercle bacillus.

Bubo and abscess also develop from other causes. They arise after the inoculation of material containing antiformin or sodium hydroxide in the strength used in the concentration methods mentioned above. This explains the necessity of neutralizing such material before it is inoculated. A similar reaction likewise frequently follows the inoculation of suspensions containing large or numerous fragments of tissue. When tissues such as the lungs or spleen are reduced to suspensions by grinding, the mixture contains a considerable quantity of tissue fragments, which, upon being inoculated, excites the production of bubo and abscess indistinguishable grossly from those produced by the other causes just enumerated. None of these early swellings have any effect upon the development of tuberculosis from the tubercle bacilli inoculated.

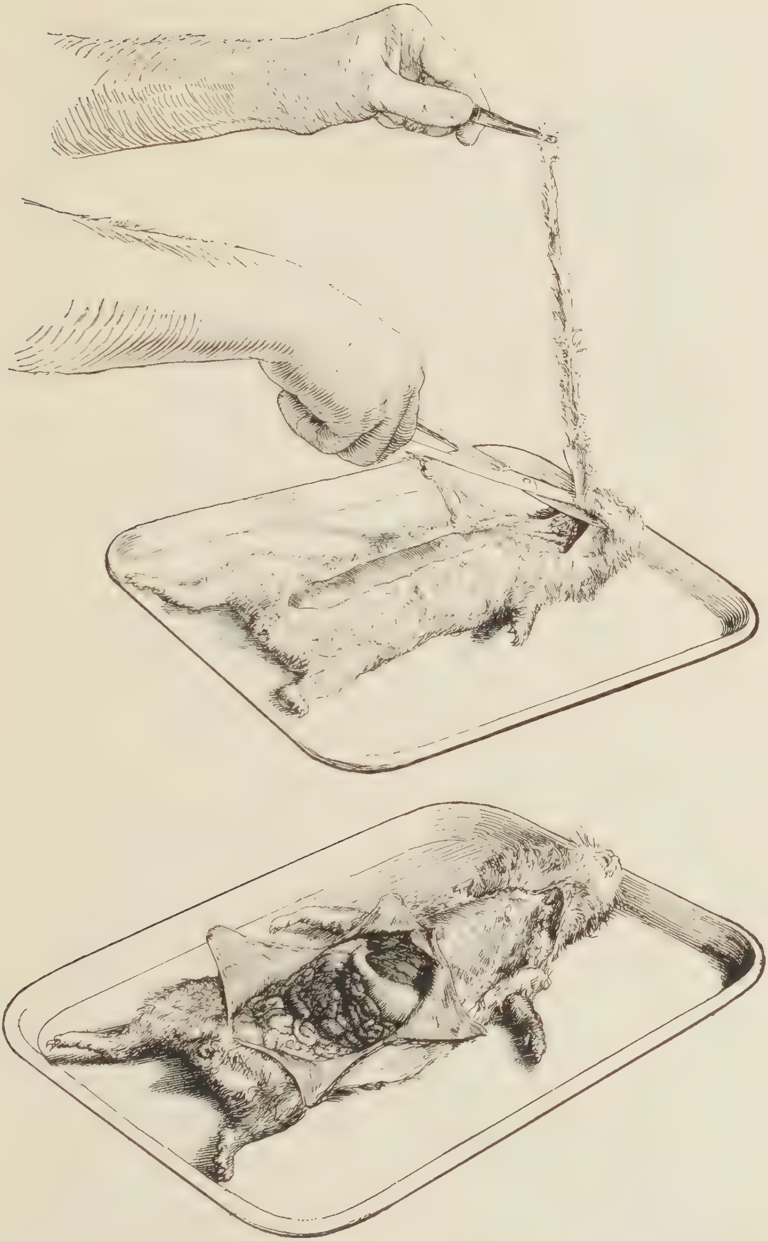
2. *After death of animal:* Definite results follow the inoculation of tubercle bacilli into guinea pigs, dosage and the mode of inoculation naturally determining in some degree the length of time elapsing between inoculation and the evolution of generalized tuberculosis. After subcutaneous inoculation in the groin, swelling and disease of the regional lymph nodes is followed by similar changes in more proximal nodes; then the spleen enlarges as also do the tracheobronchial nodes; likewise,

but later than the appearance of splenic disease, signs of tuberculosis develop in the liver and lungs. So the autopsy findings will differ in some measure, depending upon how long after infection the tissues are examined. For this reason animals should not be killed in a shorter time than six or eight weeks after inoculation unless special indications dictate otherwise.

a. *Autopsy-technique:* Animals may be painlessly killed in any one of several ways: either with an anaesthetic (chloroform, ether, illuminating gas), a sharp blow on the head, pithing, or a sudden breaking of the neck by suspending the animal from the head which is held between the fingers of one hand and giving a quick downward movement that is terminated by a jerk. There should be no unnecessary delay in performing the autopsy after death of the animal.

For doing the autopsy satisfactorily, the animal should be placed on its back on a pan with the belly thoroughly wet: dry hairs are easily carried into the body by the instruments. The animal need not be tied down. The skin is picked up by forceps in the midventral line at the level of the mammary glands and the folds cut through with scissors. The end is lifted up and the skin is cut in this manner until a streak about one-half inch wide has been cut to the chin. The skin is separated from the underlying muscle, the inguinal and axillary spaces exposed and examined. Next, the belly wall is lifted and split from the symphysis pubis to the xiphoid and the sides divided so as to form four flaps. This exposes the peritoneal cavity. The viscera may be moved from side to side so that the iliac and paraortic lymph nodes, the spleen, liver, kidneys, peritoneum, mesentery and omentum are brought to view. Careful observation of these should precede removal of the viscera. After this inspection, the oesophagus may be cut where it emerges from the diaphragm and the gastrointestinal tract removed. The diaphragm is pulled downward toward the liver in a point just ventral to the portal vein, nicked with the scissors and excised along its costal attachments. The ribs are cut near the spine and, with the sternum, are removed. The trachea is divided near the larynx and by this structure the lungs are lifted out of place, the dorsal aorta and oesophagus being cut in so doing. Such a procedure exposes all essential structures that usually contain tuberculosis. For such work large, heavy, blunt-pointed scissors of about eight inches length are desirable, because instruments of that size can be used to cut the ribs as well as the skin and do not require the hand-power that must be employed when smaller scissors are used. Several stages in the autopsy-procedure are shown in figures 20 and 21.

b. *Results at autopsy:* At six or eight weeks after inoculation the superficial inguinal lymph nodes at the site are enlarged by two, three or four diameters, are quite firm and have central caseation; the iliacs on the side of inoculation are similarly involved. The spleen, twice or thrice or even five times enlarged and of a dark color, contains numerous yellowish bosses of pinhead size which roughen the surface. Or it may be peppered with smaller, smooth, whitish-yellow areas of irregular outline lying flush with the surface. Very late in the course of the disease, the organ contains numerous large areas (5 to 10 mm. in diameter) of yellowish, granular, caseous tissue. In the liver are seen numerous yellowish-red or yellowish-green areas of tuberculosis of varying shape and size (0.5 to 5 mm. in diameter). The regional lymph node is usually enlarged and caseous. The lungs relatively escape infection. A few, as a rule not over 20 or 25 (at the most), grayish, translucent, gelatinous or sago-like areas the size of a pin's head (sometimes much smaller), sometimes with a yellowish point of caseation at the center, are usually present. In advanced disease these areas may be 5 mm. or greater in diameter and more numerous. The tracheobronchial lymph nodes become lobulated, very firm and greatly enlarged and sometimes have points of caseation. Lymph nodes in other regions of the body,—the axillaries, cervicals, superficial and deep inguinals and iliacs on the side opposite the site of infection,—also become opaque, enlarged and (very late) grossly caseous. The kidney in particular never bears any evidence of disease. A drawing of a tuberculous guinea pig is shown in figure 22. Results are so constant and so characteristic that diagnosis



Figures 20 and 21. Drawings Illustrating Two Stages in Technique of Performing Autopsy

The text carries a description of the details involved

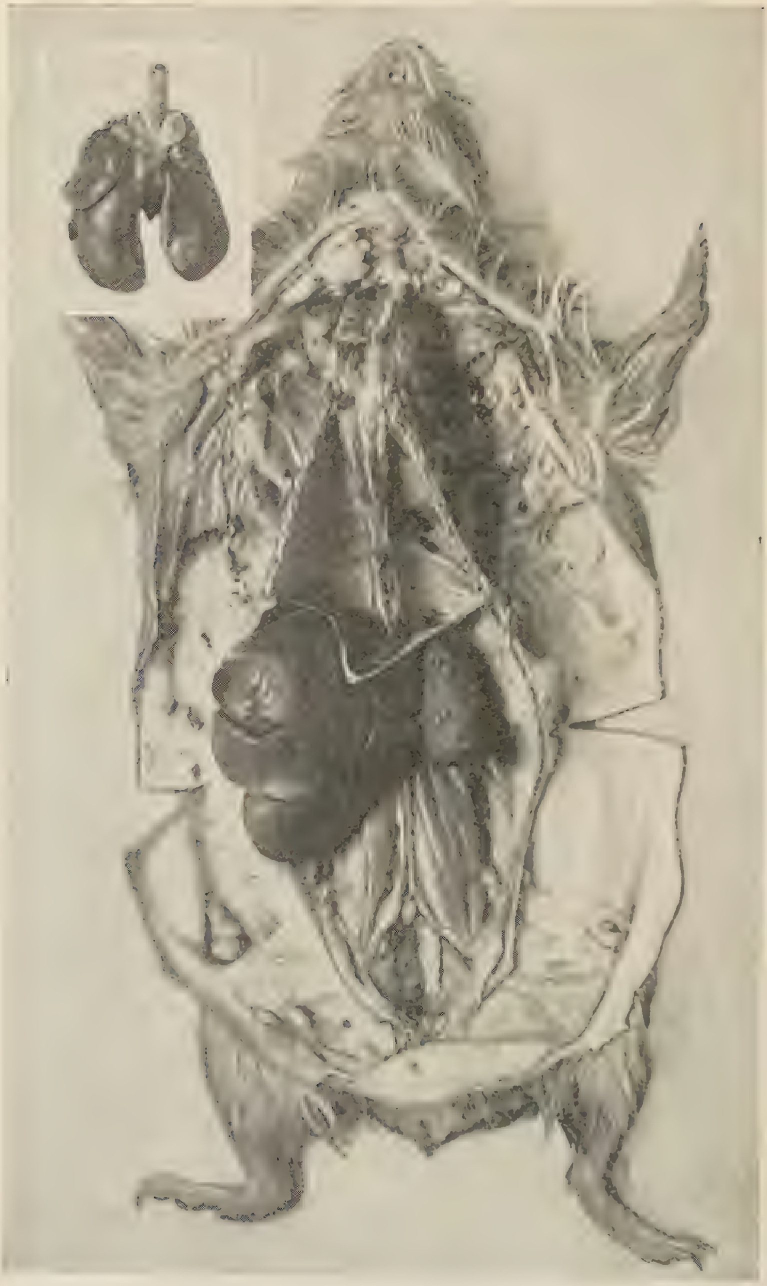


Figure 22. Halftone Drawing of Tuberculous Guinea Pig at Autopsy

An account of the findings at autopsy may be found on p. 222

may readily be made on the gross appearance without recourse to histological examination.

Biopsy with removal and smear or histological section of one of the enlarged superficial inguinal lymph nodes is sometimes resorted to in an effort to shorten the time required for making a diagnosis. But the finding of acid-fast bacilli in such circumstances does not prove that such bacilli are tubercle bacilli. If the inoculum be sputum, such evidence is dependable because it is exceedingly unusual to find nonpathogenic acid-fast bacilli in this medium. But if the inoculum were urine, for instance, the acid-fasts might conceivably be smegma bacilli, or, if it were milk, butter bacilli might be found. So demonstration of bacilli in these circumstances, without evidence of progressive disease in the animal, is not reliable

Results of Inoculation of the Rabbit

Rabbits are not used routinely for diagnostic work because of their irregular response to inoculation with human tubercle bacilli. But when inoculated with human or bovine bacilli, the rabbit at autopsy presents findings that are characteristic for each strain.

1. *Human bacillus*: Many times after inoculation with human bacilli no signs of disease are found in the rabbit, but as a rule, particularly after intravenous infection, the lungs and kidneys develop tubercle which for a time progresses but which usually undergo retrogressive changes, leaving in the surface of the kidney a few fissures or scars and in the lungs no gross permanent evidence of a previous infection. At times, however, as already mentioned, rabbits succumb to large doses of human bacilli. When this occurs no morphological difference between the diseased areas established by the two types of bacillus are apparent. (See Chapter XII).

2. *Bovine bacillus:* The smallest dose of bovine bacilli kills rabbits. Small doses produce disease less rapidly than large doses, but the animal that has been infected for six or eight weeks will in nearly every instance show abundant signs of the infection, irrespective of the size of the dose. There is usually, though not always, a soft, spreading, flabby abscess

at the site of subcutaneous inoculation in the groin, the response in the lymph nodes being nil or very slight. The iliac nodes show but little reaction and the spleen, except when recently isolated, highly virulent cultures are used, is almost invariably clean and free from gross, and usually microscopic, signs of tuberculosis. This is one of the interesting and puzzling features of the rabbit's response to tuberculous infection. However, the kidneys are nearly always involved and are usually studded with smaller or larger whitish, spherical lesions, ranging in size from one-half to 4 or 5 mm. in diameter and being elevated, dome-like, above the surface. They frequently extend, wedge-like, into the cortex and they are found only uncommonly in the medulla or calyx. The liver is not so frequently involved as it is in the guinea pig, and it is uncommon to find more than an occasional small, isolated tubercle in this organ at this stage. The lungs carry by far the brunt of the infection, regardless of the manner of inoculation. Here there is a studding with a large number of tubercles that tend to be of one size (especially if the inoculation were done intravenously) but they may vary in size from a speck at the point of vision to large caseating areas of 1 cm. in diameter or even (rarely) to almost complete consolidation of the entire organ. The areas are usually grayish-white and have caseous centers. Often they coalesce and, if the animals live sufficiently long, cavitation may develop. The tracheobronchial nodes are moderately enlarged and diseased in about one-half to three-fourths of the cases, but in a good many instances these structures are totally absent. When the inoculation is done intraperitoneally, numerous patches of tuberculous tissue and tubercles are spread over the omentum and peritoneum as well as in the lungs and kidneys. Where the animal lives a long time (3 or 4 months or longer) after inoculation, widespread lesions may develop in the eye, bones, muscles and subcutaneous tissues.

3. *Avian bacillus:* Occasionally after inoculation with avian tubercle bacilli, the rabbit quickly succumbs to a septicaemia and as a rule is dead before sufficient time has elapsed for the formation of histological tubercle. Usually no demonstrable disease develops.

Diseases Resembling Tuberculosis in Animals

There are several diseases of guinea pigs and rabbits which resemble tuberculosis. These are discussed in Chapter XIV, p. 242. to which the reader is referred.

Results of Inoculation of Fowl

Pigeons, hens and other fowl do not die from disease produced by either the human or bovine bacillus, but respond merely by formation of abscess at the site of inoculation. When, however, they acquire the avian bacillus either spontaneously in nature or by inoculation, fatal disease supervenes with considerable celerity. Where the disease becomes prevalent, flocks are decimated and caged fowl uniformly succumb. The disease is essentially visceral, involving chiefly the liver, spleen, gut and mesenteric lymph nodes, any one or all of which may become riddled. The enlargement of diseased mesenteric lymph nodes may become so marked as to greatly displace the viscera. Bacilli are eliminated in enormous numbers from the gastrointestinal tract. Calmette (31) has given a good account of the pathogenesis of avian tuberculosis.

Isolation of the Tubercle Bacillus by Inoculation of Animals

Refractory to the invasion of most of the common run of bacteria and having the capacity to clean up the inoculum by reacting permanently only to the tubercle bacillus, the guinea pig is a good vehicle for separating tubercle bacilli from contaminated material. If possible, material containing tubercle bacilli and other bacteria should be treated with antiformin or sodium hydrate before inoculation, but, if such treatment is not possible, the material may be inoculated directly. By the time generalized tuberculosis should have developed (six to eight weeks after infection) search for the bacillus in pure culture may be begun. The animal is killed, the belly shaved and made wet with 5 per cent carbolic acid. The skin is incised with sterile scissors and forceps, and, with another set of sterile instruments the muscle is similarly cut and the spleen exposed. This organ is removed and either minced and ground in a sterile mortar and the emulsified material planted on several tubes of Petroff's or Dorsct's egg media (about half of the cultures so made should grow). The spleen may be transferred to a sterile petri dish and, with sterile forceps and scissors, an area of tuberculosis removed to two sterile slides and crushed between them, the crushed material being then planted on media. In case the spleen has been contaminated, its surface is seared with a hot spatula and cultures made from tuberculous patches after crushing between slides. Other tissues than the spleen may be used, but from the lungs it is difficult to get the foci and from the abscess at the site of inoculation or the regional nodes, contaminators may still be recovered. On the media very fine granular growth begins to appear within a week or 10 days of planting. Growth is facilitated by leaving the tubes plugged but not tightly sealed for two or three days after seeding. After this time, however, they should be sealed.

CHAPTER XIV

METHODS OF STUDYING INDUCED TUBERCULOSIS IN ANIMALS

A routine tuberculosis laboratory may be used also as a research laboratory without many additions to the facilities already availing. It goes without saying that it is desirable and profitable to study the nature of disease because, while results in experimental animals cannot be carried over and applied uncritically to human disease, the basic principles behind biological reactions to infections, to irritants, to foreign bodies, are similar in the different forms of mammalian life. This is also true of the principles that underlie such phenomena as allergy and immunity in this disease. The aim and purpose in a routine laboratory should be to discover and correlate in a given case all pertinent facts available by laboratory methods as an aid to the clinician; at the same time the laboratory may be a place where the nature of the disease may be studied under controlled conditions.

GENERAL

It is naturally out of the question to run the gamut of such a subject as this and it is only in a very general way that some of the more important matters can be touched upon. The truth of this must be obvious when one realizes how many possible types of investigation there are. We must therefore confine ourselves to a consideration of some of the more common features that may be a part of almost any kind of experimental investigation in tuberculosis. Such subjects as the types of animal, strains and dosage of bacilli, anatomical methods, and some of the procedures employed in studying immunity to tuberculosis will each come up for discussion.

1. *Animals:* The question of the choice of animals for routine laboratory work in tuberculosis has already been discussed (p.70) and the same consideration holds for experimental work. For general, all around work the guinea pig is by far the best adapted of the small animals for investigation in tuberculosis. This animal reacts in about equal degree to human and bovine strains; it reacts remarkably constantly and uniformly, and invariably develops disease when inoculated even with very small numbers of bacilli. The rabbit, on the other hand, is inconstant in its response to infection with human tubercle bacilli and is unreliable in its allergic and immunological responses. It should rarely be used in work where such phenomena are being studied unless this fact is borne in mind. It is invaluable, however, as a differentiator between human and bovine strains for, as already mentioned, it responds but indifferently to human strains but succumbs to bovine strains with great readiness. It has been used extensively in studies on the bovine bacillus. Of the larger animals, cows and especially calves, have been widely used. Cobbett's book (41) carries a good account of infection, dosage, course of the disease and findings at autopsy in a large number of experimental animals, particularly the larger ones.

a. *Care of animals:* Loss of animals in experimental work is costly both because of the expense of replacing the animals and, much more, because of the frequent undoing of an experiment. Occasionally an epidemic of pneumonia, snuffles or diarrhea (?) seems inevitable, but the likehood of these is greatly lessened where animals receive adequate care. Animals should go in quarantine for two weeks when they are first brought in. It is best to have a small room, separate from the regular animal room, where all incoming animals may be housed for the quarantine period. Animals should be kept in cages with movable bottoms so that both the cage and its bottom can be emptied, sterilized (either in a vat of carbolic acid or other disinfecting fluid or in a steam sterilizer) and scrubbed with a brush and hot water. And every cage containing animals should receive this treatment at least once a week. Scrupulous attention to such matters should eliminate the so-called "spontaneous" tuberculosis in laboratory animals. Using this care, with an annual turnover of six or seven hundred animals, we have seen no certain instance of spontaneous tuberculosis in ten years.

While passage of bacilli from infected to normal animals resulting in disease of the latter is very rare, it is not an impossible occurrence, and normal and infected animals should never be caged together. Likewise, animals in one experiment should never be caged with those in a different experiment. Sexes should be kept separate.

The food requirements are relatively simple. There should always be an abundance of hay in the cages both for ruffage and for bedding. Feeding should be done twice a day. Freshly wet oats and greenstuff, such as lettuce or cabbage, and hay should be given in the morning and freshly wet oats and hay in the afternoon. If oats is allowed to soak for more than a few hours, it may produce a fatal diarrhea in the animals. With this danger guarded against, however, oats makes an excellent food that is both inexpensive and adequate to the animals' needs.

Marking animals with numbered tags or by nicks in the ear is often done but is unsatisfactory since such markings may be torn away when animals fight. It is nearly always possible to describe animals, especially guinea pigs, by their color and color distribution—many albinos, for instance, have one or more dark ears or feet. A patch of distinguishing color somewhere about most of these animals will enable one to adequately describe them, and it is unusual to have to resort to marking with dyes. It is more difficult in the rabbit, but here a smudge of carbolfuchsin on one ear or on the side will serve as a distinguishing mark. This should be renewed every month, however.

2. *Strains of tubercle bacilli in common use in the study of tuberculosis:* Ordinarily, variation in the virulence of a strain of tubercle bacilli from time to time is exceedingly slight. Likewise there is but slight difference in the virulence of different strains, for by far most of the strains that are isolated possess a degree of virulence that is quite similar and uniform and one

that is also high. Griffith (76), however, has recently isolated several strains with a comparatively low virulence. A great deal of work in tuberculosis in this country has been done with the use of a few well known strains that came originally from Saranac Lake. Baldwin (11) recently gave a brief, lucid account of several of these.

The most important of the strains in common use is the so-called R1, which is mentioned earlier (p. 85). This strain, Trudeau isolated in 1891 from the lungs of a man who had died of miliary tuberculosis. It was normally virulent for laboratory animals for a few years when its virulence began to taper off markedly. And now for more than thirty years the strain has maintained a capacity to infect animals and to produce tissue reaction in the animal-body, such as enlargement and, at times, caseation of lymph nodes near the site of inoculation, an occasional, very small, elevated tuberculous area of 1 mm. or less in diameter in the spleen or lung, and enlargement of the tracheobronchial lymph nodes with histological tubercle. Animals seldom if ever die from infection with R1 bacilli, and they have been kept for more than four years after infection, the intensity of the latter becoming gradually less. It has been shown by Krause and Willis (116) that repeated daily inoculations of very large doses fail to produce any reaction that resembles that of progressive tuberculosis in animals. It establishes infection and thus sets up the allergic and immunological phenomena associated with infection without causing progressive disease. It grows well on any of the usual media.

Another human strain, known as H37, was isolated in 1905 from human sputum also at the Saranac Laboratory.[21] It is pathogenic for guinea pigs and, although animals infected with it may live longer than did animals infected with it 10 years ago, it is uniformly and constantly pathogenic, always producing disease that is progressive and lethal within four months of infection. Several other human strains (H36, H39) are also used but H37 is a sort of standard.

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[21] Cultures of this strain have twice within the last year or two played the strange trick of completely losing their virulence, and in at least one of the instances there was an associated loss of tuberculogenic properties (as determined grossly). The culture at the Saranac Laboratory first went through this change, but those in our laboratory continued to run true to form and we supplied that laboratory with our culture. A year or so later we had precisely the same experience with our cultures and obtained from the Saranac Laboratory cultures that appeared normally virulent. We are as yet totally in the dark as to the meaning or explanation of this most interesting occurrence.

A bovine strain, isolated in 1902 from a case of spontaneous tuberculosis in a cow and known as B1, is the most widely used bovine strain in this country. Like the others, it also came from Saranac Lake. It possesses a moderately high virulence for rabbits and guinea pigs.

Many other human and bovine strains are in use in laboratories throughout the country. Results from infection with them are undoubtedly comparable in most points with those produced by the strains just described. It is because these three strains are widely used and are "standard," as it were (especially R1) that an account of them has been given. Everything considered, it would seem desirable to use these strains in experimental work unless some reason to the contrary should exist, because they are tried; they are constant and produce very uniform results as a rule, whereas new strains should be pretty well observed before being used for experimental purposes.

Several strains of avian tubercle bacilli are in use.

3. *Standards for estimating pathogenicity:* At best, the standards on which virulence is estimated have not been free from much justifiable criticism, for the difficulties are many. Baldwin, in the paper just referred to, has called attention as follows to some of the factors to be evaluated: Factors on the part of the bacillus are "(a) date of isolation of the culture from the patient; (b) age of the culture used for inoculation; (c) nature and reaction of culture medium; (d) part of growth selected for inoculation; (e) number of agglutinated or dead organisms included; (f) fluid used for suspensions; (g) dosage. On the part of the animal one should consider (a) race, age and weight; (b) sex; (c) site and method of inoculation; (d) amount of trauma; (e) feeding and isolation of animals; (f) intercurrent

diseases; (g) natural susceptibility to tuberculosis of the three main types."

This author laid down details of a procedure to be followed in preparing the suspension and inoculating and sacrificing the animals. He recommended that the findings at autopsy be evaluated on the basis of a constant inoculation-procedure such as the one shown in the accompanying statement.

"1. Take a spadeful of bacilli from the thin edge of a freshly grown culture grown at least two weeks on plain egg tubes (Dorset's medium or Petroff's egg medium).
2. Rub in a glass mortar with ground glass pestle with three to five drops of sterile plain broth or bile; dilute to 10 cc. with the same broth.
3. Centrifuge in a straight-sided tube (International size I-B) at lowest speed for two minutes. The speed averages 850 R.P.M.
4. Pipette off the top half and stain a 2 mm. loopful, first fixed with albumin on a slide.
5. If clumps of four to ten bacilli or more are seen, centrifuge two minutes further.
6. The suspension should contain clumps of not more than two bacilli and singles averaging three bacilli to a field; if more numerous, dilute proportionately.
7. The suspension, separately measured in doses of 0.1 cc., should be freshly mixed for each inoculation and injected subcutaneously in the right groin of ten male guinea-pigs averaging 350 gms. weight.
8. Animals should be killed at the end of fifty days and lesions compared and recorded as follows by macroscopic appearance:
   1st degree: Disease of regional nodes only.
   2nd degree: Disease of regional nodes and spleen or liver.
   3rd degree: Disease of regional nodes, spleen and liver.
   4th degree: Disease of regional nodes, spleen, liver and lungs; or caseation of spleen or liver without lung in-involvement.

"The object will be to introduce a minimal dose capable of infecting without the foreign-body effects either from large doses or from dead bacilli or clumps. In this way only would guinea-pigs reveal differences, because large doses may mask them. The importance of a time limit is also due to the unequal spread of the disease during the early weeks, but a gradual approach to similarity after six to twelve weeks."

Duration of life after inoculation with bovine tubercle bacilli is generally somewhat shorter than that after inoculation with human bacilli. But variation in the time of death of animals is fairly wide and quite unreliable unless comparatively large numbers of animals are used. Numerous factors, such as the adequacy of housing and feeding and the occurrence of intercurrent infections, contribute toward making undependable the reckoning of virulence on the span of life after infection. These factors also may influence the evolution of the disease but to a less degree than they do the length of life. A more reliable standard may be had if one infects a group of animals and, after the lapse of a given time, sacrifices them all and compares the results, as recommended by Baldwin(11).

DOSAGE

The size and the time relationships of dosage are matters of the greatest importance in experimental tuberculosis and are factors that are altogether too often lost sight of, so that a great deal of work is open to serious criticism on this score. It may make a material difference in the result obtained after inoculation whether the animal was given a large dose or a small one, whether dosage was single or repeated, whether the time interval between doses was hours or days, whether there were large clumps of bacilli in the suspension used for inoculation. It is remarkably easy to produce infection in susceptible animals, but to inoculate in such a way as to be able later to observe the finer and more important features of the developing infection is not quite so easily accomplished. A single inoculation of a given dose of a given strain of bacilli produces uniform results by and large. Repeated doses totaling a smaller amount may produce more marked lesion. If the second inoculation is made as late as two or three weeks after the first, the ultimate results from the second one are likely to be much less severe than would have been the case had the second inoculation followed the first by only a few days. More will be said about this matter, however, when we discuss immunity. Bacilli may be carelessly made into a suspension and inoculated. But the large clumps of bacilli in such a preparation will arouse a different initial reaction in the animal from the reaction to single bacilli. These latter stimulate the body to proliferative reaction with tubercle-formation, but the former elicit, in addition to tubercle-formation, a reaction characterized by some degree of inflammation with a corresponding difference in histological appearance. It is therefore of importance to carefully prepare bacilli for inoculation and to carefully estimate not only the size of the dose but the degree of bacillary suspension as well. A salt solution-suspension of bacilli, prepared in the manner described below or by Baldwin, produces uniform results when inoculated, and is recommended. There are no clumps to act as foreign bodies.

1. *Preparation of suspensions:* Bacilli are much more satisfactorily handled when suspended in solutions than when they come directly from the tube, for, in the latter state they stick together in clumps and in suspensions they should be clump-free. For making suspensions, a mortar and pestle should be sterilized with heat or treated for five minutes with sulphuric acid-potassium bichromate cleaning solution,[22] washed with tap water, rinsed with physiological saline and drained free of as much of the last solution as can be poured or shaken out. Then bacilli are transferred to the mortar and ground for about five minutes—until the substance comes to have the consistence of thick, uniform, homogeneous paste. During the process it is sometimes necessary to add a drop of the salt solution to prevent drying and to facilitate grinding. At the end of the grinding, physiological saline is added slowly, drop by drop, agitation or stirring with the pestle being continued until 1 cc. has been added. After this point the

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[22] Cleaning solution may be prepared as indicated on page 3.

addition may proceed more rapidly. Rapid addition of water at first causes an agglutination of bacilli and makes it impossible to produce a homogeneous suspension. Too much grinding destroys some of the bacilli. Bacilli in suspension tend to agglutinate if the latter is allowed to stand for longer than 10 or 15 minutes. Suspensions should therefore be gently agitated every few minutes. Jennings (90) has found that agglutination is obviated if one uses fifteenth normal Na_2HPO_4, which is isotonic with pH of about 8.7, instead of physiological salt solution in preparing the suspension.

2. *Standardization of suspensions:* The approximate strength of a suspension may be estimated either by determining the number of bacilli to the oil immersion field, as follows, or by weighing bacilli as they come from the culture.

a. *Counting method* (stained preparation):

Cover a slide thinly with serum as a fixative.
With a small 2 mm. loop, transfer a small drop of the suspension to the slide.
Indicate the area with a wax pencil on bottom of slide
Allow drop to dry at room temperature or in an incubator.
Fix with gentle heat.
Stain for tubercle bacilli.
Examine and determine the number of bacilli to the oil immersion field.

The most carefully prepared suspensions will contain many small clumps of bacilli and these may be removed by centrifugalization, by filtering through a thin layer of cotton and by dilution. The centrifugalization is always a necessity and the other two means are frequently very useful. Several hours may be required to properly prepare and standardize a suspension. An apparently satisfactory method of counting the bacilli in a suspension has lately been devised by Jennings (90).

b. *Weighing method:*

Strength of doses is also determined by weighing

> bacilli as they come from the tube and then grinding and carefully adding a measured amount of physiological saline. Thus the dosage per unit of suspension is known.

While the weight-method is quite satisfactory for certain purposes, there is an obvious objection to it when used for certain other purposes, although it is often used. This objection is the presence of clumps in the suspension, and may be offset in the manner described below. The suspension is prepared as outlined, centrifugalized at moderate speed for two minutes to eliminate the clumps, and 1 cc. of the supernatant fluid is placed in each of two watch crystals that have just been very carefully weighed. The material in the crystals is dried and weighed, and deduction is made for the sodium chloride. This will give the amount by dry weight of bacilli to the cubic centimeter of suspension.

3. *Quantities for inoculation (dosage) in guinea pigs:* Either of the methods mentioned above for determining dosage may be used, but we have come by habit to use the "bacilli to the oil immersion field" method rather more for guinea pigs and the weight method for rabbits. Dosage (as determined by either means) for the various types of inoculation and results will be considered below.

a. *Subcutaneous inoculation:* The technique employed in the various types of inoculation has been described in Chapter XII. The actual dosage may vary considerably, depending upon the end in view. In general, animals succumb more quickly to intravenous or intraperitoneal than to subcutaneous inoculations of the same dose, so that subcutaneous dosage is usually larger. Subcutaneous administration of 0.1 mgm. of human tubercle bacilli in the guinea pig induces marked, generalized tuberculosis by the sixth week and usually produces death before 10 weeks: One milligram will produce widespread lesion and death a little earlier than the smaller dose. Infection with 0.1 cc. of a carefully made suspension that is without clumps of more than two or three bacilli and has from two to five bacilli to the oil immersion field will produce generalized tuberculosis in about six or eight weeks and death of the animal usually within 12 weeks. This is a good, average dose for work where not too fine differences are sought in results. A suspension without clumps and with an average of one bacillus to every three or four oil immersion fields is entirely adequate to produce tuberculosis after an inoculation of 0.1 cc. Lesion is widespread and fairly well developed within eight or nine weeks. Doses may range the scale between or on either side of these two latter suspensions.

b. *Intravenous inoculation:* In general this type of inoculation is not used very much in guinea pigs. Dosage larger than 0.1 mgm. or 0.1 cc. of a suspension containing two or three bacilli to the oil immersion field need never be used. The presence of clumps is of great importance.

c. *Intraperitoneal inoculation:* The merits of this type of inoculation have been discussed. The dosage is usually intermediate between that for subcutaneous and intravenous inoculation.

d. *Other methods of inoculation:* Dosage in these will vary in accordance with the mode and the purpose of the inoculation. With the average subcutaneous dosage given, the size of the dose by other modes may be satisfactorily determined.

4. *Quantities for inoculation (dosage) in rabbits:* The rabbit is exquisitely sensitive to bovine tubercle bacilli. Griffith (74) and Cobbett (40) have each shown that half-grown rabbits develop generalized tuberculosis and die in about 10 weeks from intraperitoneal inoculation of 0.00,000,01 mgm. of bovine bacilli and that young rabbits die with the same picture after similar inoculation of 0.00,000,000,01 mgm. of this strain. Rabbits may be inoculated in a variety of ways and the accompanying table (table 20) will show the approximate dosage usually employed in the different methods of inoculation.

Since a dose of 0.01 mgm. intraveneously of bovine tubercle bacilli always produces fatal results in rabbits and the same dose of human strains is never fatal, Cobbett has suggested that this be the standard dosage for typing unknown strains. If intraperitoneal inoculation be used, 1.0 mgm. of bovine or 10.0 mgm.

of human would be a standard dosage as the former always kills and the latter never does. Likewise the standard dose in subcutaneous inoculation should be 1.0 mgm. of bovine strains and from 10 to 100 mgm. of human strains.

TABLE 20

(See Cobbett (40))

	Bovine infection		Human infection	
Type of inoculation	Dosage	Result	Dosage	Result
Intravenous	0.01 mgm.	Extensive disease. Death in 8 to 10 weeks.	0.01 mgm.	Slight, regressive disease. Never death.
	0.1 mgm.	Rapid death in 3 to 5 weeks.	0.1 mgm.	Slight or no disease in about 65%. Death in about 35% within 3 or 4 months.
			1.0 mgm.	Disease and often death.
Intraperitoneal	0.1 mgm.	Extensive disease. Death in about 6 weeks.	10.0 mgm.	Occasional disease that is ultimately regressive.
	1.0 mgm.	Rapid death in 3 or 4 weeks.	50.0 mgm.	Progressive disease and occasional death.
Subcutaneous	0.1 mgm.	Progressive disease ultimately fatal.	Very large	An occasional slight lesion—nonprogressive.
	1.0 mgm.	More progressive. Death in 2 or 3 months.	Up to 100 mgm.	Never death from tuberculosis.

Response to Inoculation

Tubercle bacilli may initiate disease once they get within the body, regardless of the manner or site of their introduction. It seems established, however, that neither epithelium nor endothelium (except possibly in embolism of capillaries (?) with clumps of bacilli) are capable of reacting to the bacillus. It appears that the microörganism must be carried through these tissues to the structures beneath or back of them (and thus to the domain of the lymphatics) before reaction occurs and lesion is established. This is true of the skin, lungs, intestines and probably the other viscera. When epithelium or endothelium become involved, it is almost certain that the involvement comes about from encroachment of progressive tuberculosis rather than that these tissues reacted to the bacillus to form initial tubercle.

Distribution of Lesion in the Two Animals

1. *Guinea pig:* In the guinea pig, it is consistently the lungs that are spared until late and the spleen, tracheobronchial nodes and very often the liver that are most involved after infection. Little or nothing is found in the kidney. A more complete description of the findings in the guinea pig is given on page 222.

2. *Rabbit:* Regardless of the manner of inoculation, the rabbit's lungs and, to less extent, kidneys always bear the brunt of the infection while the liver and spleen are relatively spared. Three or four weeks after intravenous inoculation with bovine tubercle bacilli or a little later than this after intraperitoneal or subcutaneous inoculation, the lungs are voluminous, rubbery, only partially collapsible and are studded with grayish-white or whitish-yellow areas of tubercle varying in size from a tiny speck to a diameter of six, eight or even 10 mm., and these may often be caseous at the center. Later they become greatly enlarged, and an entire lobe may be consolidated. The tracheobronchial nodes are rarely involved to any great extent. The kidneys have numerous circular, discrete, distinctly spherical elevations measuring up to 5 mm.

embossed on the surface and extending, wedge-like, into the cortex. They occasionally involve the calyx and medulla. It is unusual to find even an occasional, very slight lesion in the spleen although the liver may develop a goodly amount of disease. In chronic cases, there is an extensive seeding in many parts of the body including, in addition to the viscera, muscle, bone, joint, mammary glands, testes and eyes. Lesions initiated by human bacilli almost never become so widely distributed or so extensive, but at times from these strains little areas of caseation 5 to 8 mm. in diameter may form in the lungs.

The probable explanation of differences in pulmonary involvement in these two animals has been offered by Krause (104) on the basis of Miller's anatomical work and is the most satisfactory one yet advanced. It places the issue largely on the basis of differences in the finer anatomy, especially of the lymphatics and lymphoid tissue, of the lungs in the two. The lungs of the rabbit, as has already been pointed out, possess relatively small lymphatics and a very large amount of lymphoid tissue which may readily act as mechanical filters and hold bacilli, while in the guinea pig's lungs the lymphatics are abundant and large and the intrapulmonary lymphoid tissue scant. In the lungs of the former the infection "sticks"; in those of the latter it tends to pass through to be caught up in the tracheobronchial lymph nodes. That anatomical conditions explain the differences in infection in spleen and kidney in the two animals, however, lacks histological confirmation as yet.

Spontaneous Diseases Which Resemble Tuberculosis in Experimental Animals

Spontaneous tuberculosis in rabbits and guinea pigs in their native haunts is exceedingly rare if it ever occurs. It is also very rare in well kept laboratories. The possibility of an occasional, accidental infection always exists where large numbers of infected animals, some of them with open ulcers at the site of inoculation, live in the same animal rooms though not necessarily in the same cages, with normal animals. But I have never seen a certain, undoubted case.

There are, however, a few diseases which closely simulate tuberculosis in their general gross appearance. They are coccidiosis and chronic abscess in rabbits, and the so-called "pseudotuberculosis" and chronic pneumonia in guinea pigs. They will be described briefly.

1. *Coccidiosis:* Coccidiosis, a disease caused by the sporozoan parasite, *coccidium cuniculi*, is endemic among rabbits. It may infect rabbits for a long time without apparent injury to health, although it is a frequent cause of death. This disease superficially resembles tuberculosis in the gross, but the lesions are of a whiter, less cream-colored appearance than the lesions of tubercle; they are softer and are somewhat watery; they are more discretely circular and do not have caseating centers. They are usually confined to the liver, or, if the disease is extensive, this organ bears the brunt. They may obscure tuberculous lesions. The nature of the lesion is determined in a moment if one gently squeezes a little bit of one of the lesions between two slides and observes it with the low power objective of the microscope. The parasitic ovae are readily seen.

2. *Chronic abscess:* The rabbit is very prone to develop indolent, chronic, subcutaneous abscesses which frequently simulate closely the ordinary "cold" abscess in man. They contain yellowish-white, thick, sometimes inspissated or granular pus. The latter is occasionally free from bacteria upon stain and sterile upon culture, but it frequently yields a few colonies of strepto- and staphylococci. The abscesses may be large or small but usually do not measure more than 8 or 10 mm. in diameter. They generally produce a reaction in the regional lymph nodes (superficial and deep inguinals, axillaries) that is characterized by firm enlargement and, later, abscess-formation. Grossly, these nodes may very strikingly resemble tuberculosis. But the local nature of the reaction (generally) and the absence of signs of generalized disease, such as would follow the inoculation with bovine tubercle bacilli, give the cue to the true nature of the structures. They should not be con-

fused with infection with human tubercle bacilli for, when rabbits are infected with this type of bacillus, the lungs rather than the lymphatic system carry the marks of the disease, if indeed, the animal reacts at all.

These abscesses sometimes involve the testicles and (less commonly) the mammary glands, but *tuberculous* orchitis and mastitis are only unusually encountered in rabbits until late in the course of the disease, when the process is far advanced and generalized. Such abscesses probably have their origin in infections which follow scratching, biting and fighting among cage-mates (169).

3. *Pseudotuberculosis:* The disease called "pseudotuberculosis" occurs frequently in normal guinea pigs in some parts of the world, but it is not very commonly encountered in this country. Its lesions are found largely in the spleen, liver and mesenteric lymph nodes. They are small, quite round and white and superficially resemble those of tuberculosis very much. The regular and constant roundness of the affected areas constitute the most conspicuous feature of the disease, and is of great help in distinguishing the condition from tuberculosis. The disease is produced by a nonacid-fast bacillus belonging to the group of septicaemic bacteria of which the bacillus of oriental plague is a member. Smear and stain will readily reveal the true nature of the disease. When experimentally inoculated, the bacterium sets up disease which, except for the rapidity of its development, strikingly resembles tuberculosis, having necrosis, involvement of regional lymph nodes and the general distribution of the latter disease. Animals with the disease very commonly live a healthy life, but it spreads rapidly through the very young.[23]

4. *Chronic pneumonia:* Although chronic pneumonia in guinea pigs usually runs true to the characteristic appearance, one encounters an occasional instance in which the disease, apparently very chronic, resembles somewhat an extensive tuberculosis of the lungs. Yellowish-gray, circular or nodular areas may be seen lying in close proximity to each other and

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[23] Cobbett (41) has given a good description of this disease.

at times involving large portions of the organ. These are frequently either surrounded by patches of characteristic pneumonia or are "set" in areas of pneumonic consolidation. They are not elevated above the surface and are not gelatinous in appearance, and these two features are of a great deal of help in the differentiation. Frequently some caseation may be seen toward the center. They are present in the depths of the lung as well as in the pleura. On section they are seen to have central necrosis surrounded by a great deal of fibrous tissue. No epithelioid cells and no true structural tubercles are apparent. Sections contain cocci of undetermined classification but no tubercle bacilli.

Anatomical Methods

1. *Injection methods*: The relations of tuberculosis to the vascular and air passages in the lungs are often brought out with great clearness by injection and corrosion methods. These are numerous. For several that I shall mention, I am greatly indebted to Dr. W. S. Miller, in whose hands this particular type of investigation has been exceedingly fruitful. Injections of the lung are regulated by choosing an injection mass which has granules large enough to plug the capillaries or small enough to pass through them so that one may inject arteries, for instance, with one color, and veins and capillaries with another. The procedure is subject to variation in several ways. Ordinarily, the injection masses are mixed with pigments which both give the color to the mixture and the size to its individual granules.

Injections are usually made with a pressure pump that is attached to the water pipe at the sink. A mercury manometer is connected to the pump and from it a rubber tube leads to the bottle containing the injection fluid and thence to canulae which are placed in position in the trachea, heart, or great vessels.

In making an injection, the animal is killed either by bleeding or by anaesthetization with chloroform, ether or a mixture of ether and amyl nitrite. The bleeding may be done either by severing the femoral artery and vein while the assistant holds the animal in a vertical position, or by first administering ether and nitrite and then opening the abdominal cavity and cutting the abdominal aorta or by opening the thorax and cutting off the apex of the heart. After the death of the animal, the heart is exposed through abdominal approach by excision of the diaphragm and removal of the ventral wall of the thorax. The apex of the heart is cut off so that both ventricles are opened. With a curved forceps a double ligature is placed loosely around the aorta and pulmonary artery as they lie together, and another is laid around the pulmonary veins. A canula is introduced into the right ventricle, through the auricle and into the pulmonary artery and is tied in place by the ligature, the ends of which are now brought up and tied around a short rubber tube connected to the canula. This secures the canula. Another canula is similarly placed in the left ventricle and tied in the pulmonary veins at the point where they reach the heart. The tube leading from the manometer passes to the bottle containing the injection mass. Stoppers must be tied into the bottles. Large artery clamps should be used to clamp off the tubes when p essure is not being used, because in this way the water flow that is producing pressure at a constant level need not be touched. It is sometimes desirable to do injections simultaneously in both arterial and venous systems. To do this, a glass tube in the form of the letter Y is interposed in the system and two bottles are attached. The glass Y tube may be made with three leads to as many bottles.

Before injection, the blood vessels should be washed out with physiological saline containing 2 per cent sodium citrate. The injection mass should be introduced into the canula only after the latter has been filled with acetone, and no air should be admitted. In this way the resulting corrosion will be free from small holes. Pressure may be turned on in one system and the injection mass allowed to flow for a few seconds before it is applied on the other side, and thus variations in the extent of the injection on one side or the other may be obtained. A pressure of more than 60 mm. of mercury should rarely be used intravascularly if the tissues are to be studied histologically for fine divisions. A pressure of more than 40 mm. in the air passages is likely to cause a rupture. But with thicker masses, as starch or celloidin, pressures must be considerably increased. Since these masses are used only for corrosions or for dissection of the vessels larger than capillaries, rupture in these circumstances is usually not of such great moment.

Injection of the bronchial arteries may be done in either of two ways. By one scheme the great vessels of the neck are obliterated by a strong, very tight ligature around the neck, the aorta is ligated just after it leaves the heart, the canula tied in the abdominal aorta just below the diaphragm and injection thus made through the bronchial arteries which ordinarily arise from the upper intercostals. In such an injection care should be observed to split the sternum in the midline, not quite to the suprasternal notch, to avoid leakage from severed intercostal or mammary vessels. By this means, the bronchial arteries are injected, but some of the injection fluid passes out into the capillaries, veins and arteries of the lung. The other way consists in injecting either into the veins with the arteries ligated or into the arteries with the veins ligated. High pressure—100 to 140 mm. of mercury—must be maintained sometimes for as long as 10 minutes when bronchial arteries are injected. Usually the injection is good in the bronchial arteries only, for in the rest of the lungs the injection, because of rupture from the pressure, is so dense and diffuse as to obscure much of the structure.

When gelatin is used, the mixture must first be thoroughly melted and well shaken, and during the injection the bottle must be kept in a deep pan containing water heated to 50°C. The lungs and thorax of the animal must also be submerged in warm water. It is desirable though not necessary that the lung should be maintained in distension at a constant pressure of 10 mm. of mercury (through a canula in the trachea) while the injections into the arteries and veins are being made. After injections are completed, the pressure is slowly released, vessels ligated and alcohol, Zenker's fluid or some other fixative is allowed to flow into the trachea by gravity (25 or 30 cc. is average for the guinea pig, 60 cc. for the rabbit), the trachea is next ligated, the entire thorax (or just the lungs if these have been previously removed) put in a beaker containing fixing fluid and covered with cotton and the beaker placed in the ice-box for several hours to allow the gelatin to harden.

The duration of injection is a relative matter and must be worked out for the thickness of the mass, the type of animal, etc. After the injection, fixing fluid may be introduced into the trachea, as already indicated, but care must be exercised here, as also in the injection, that the lungs are not distended to a larger volume than would be the case if they were in the closed thorax. If injection is done for corrosion, pressure should be discontinued as soon as the mass is visible at any point in the pleura.

a. *Injection masses:* These may be thick or thin and will be different for different purposes. A few of those more commonly used will be given. For more complete data on this subject, the reader is referred to Lee's Vade Mecum (122).

1. *Blue gelatin:*

Gelatin (for culture media)............ 50 gm.
Distilled water......................250 cc.

Bring water to a slow boil and continue the boiling until gelatin is dissolved, stirring frequently.

Prepare a saturated aqueous suspension of Prussian blue pulverized to impalpable granules[24]. 250 cc. (If blue does not go into suspension readily, add a few drops of weak oxalic acid).

Heat: and slowly pour the blue into the hot gelatin, stirring continually.

Filter through gauze into the bottle used for the injection.

Allow to cool and add crystals of thymol as a preservative.

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[24] The Coleman and Bell Company puts out a blue powder known as "Tieman's Soluble Blue" which is excellent for this purpose. It is insoluble and does not stain the tissues and is such a very fine, impalpable flour that granules are scarcely discernable under the microscope.

This preparation is practically granule-free and it flows quite freely through the pulmonary capillaries. Ultramarine blue may be used instead of the other dyes mentioned, but its solubility must be tested with 70 per cent alcohol. It is difficult to get a good ultramarine blue at the present time.

2. *Red gelatin:*

Gelatin	50 gm.
Distilled water	250 cc.

Bring to a slow boil and continue boiling until gelatin is dissolved, stirring frequently. While hot, stir in Chinese vermilion until a deep red is obtained and until further additions no longer produce a change in the color.

Filter through thin gauze into injection bottle. When cool, place a crystal of thymol on top.

Red gelatin may also be prepared in the same manner with red oxide of mercury, cinnibar, or carmine, but with the latter the reaction of the mixture is of importance. Cinnibar or mercuric-sulphide is much coarser than vermilion (which is finely ground cinnibar) and constitutes a very valuable medium for injection where it is desired to inject either arteries or veins but not capillaries. These preparations must be well shaken or mixed before use as the pigment is heavy and settles rapidly to the bottom of the bottle.

When the red and blue gelatin have been injected into the two blood systems and the lungs fixed through the trachea, thick, freehand sections may be cut and cleared by the Spalteholz method (p. 271). Such preparations are sometimes very beautiful and relationships of infected areas are clearly shown. When clearing is desired, however, formalin in 10 per cent strength is the fixative of choice. Such tissue, of course, is subject to ordinary section cutting but must be imbedded in celloidin rather than paraffin because heat is necessarily employed in the latter technique and this will cause the gelatin to melt.

3. *Starch*: For certain studies where tissues are not to be sectioned, corn starch makes a very satisfactory injection mass.

It is mixed in 70 per cent alcohol in varying amounts, depending upon whether the mass should be thick or thin; that is, whether one desires to inject only the large vessels or the finer ones as well. Pigments such as vermilion or ultramarine blue are mixed into the suspension until the desired color is obtained. Such a mass is particularly useful for injection of vessels (that is, such as the bronchial arteries), prior to a dissection of these structures. Miller (149) has made use of corn starch as a differential injection mass for stereoroentgenograms and has taken advantage of the fact that vermilion in the starch suspension casts a different shadow in the X-ray from ultramarine blue. The pulmonary artery may be injected with a starch suspension containing vermilion and the veins with a suspension of starch and ultramarine blue. In the procedure the pressure requirements are sometimes considerable. "If only the large vessels be desired, a mass that will flow with a pressure of 100 mm. of Hg. will give the desired results (in the dog); but if the finer vessels are to be injected, the mass must be diluted with 70 per cent alcohol and the pressure increased." According to Miller, no fixed rule can be laid down for the proportions of the mixture, and each new package of starch or supply of pigment must be tested on animals first and the mass must be thus standardized.

b. *Injection fluids:* For certain purposes thinner fluids rather than injection masses are desirable. A few are given below.

1. *India ink:*

India ink and distilled water āā, or,

India ink	75 parts
Distilled water	25 parts

Either of these strengths makes a satisfactory injecting fluid for blood vessels.

2. *Gerota's (68) blue fluid for lymphatics:*

Berlin or Paris blue	5 gm.
Turpentine	5 cc.
Ether	20 cc.

The blue comes in collapsible tubes as a paste thoroughly mixed in oil. This is ground in a mortar with the turpentine. The ether is then added. This mixture is quite thin and may be injected into very small vessels.

Another very satisfactory fluid may be prepared by grinding 5 gms. of Tieman's Soluble Blue in a mortar with a few drops of physiological saline, adding gradually just a little of the dye and saline at a time and continuing the grinding until the dye becomes pasty; sufficient physiological saline is then added to bring the total to 25 cc.

2. *Corrosion methods:* Corrosions or casts of the blood systems or air passages may be prepared by injecting Wood's metal, celloidin or other nondigestible materials and then subjecting the lungs to the digestive action of hydrochloric acid and pepsin. Anatomical relationships are well shown in this way. There are two general ways of making corrosions: the more commonly used consists in introducing the mass into the lung in its fresh state, usually in situ; by the other the lung is distended to the volume it occupies in moderate expansion in the chest, is fixed and dried in this state and injected. The latter method is infinitely more tedious but the results obtained are often very gratifying.

In the first-named method, the general principles governing injections are employed. Methods are different for celloidin and Wood's metal.

In the dry method, the lungs are very carefully removed from the thorax so that there are no nicks or no areas of atelectasis produced by the touch of the instruments. With the trachea attached to a canula, the lungs are carefully placed on a warm sand bath. Very gradually pressure is raised to 10 mm. of mercury, the lungs being distended at this pressure to a size approximating that of normal inspiration. If atelectatic areas are present, a pressure of as much as 35 mm. of mercury may sometimes be necessary, but this should be reduced to 10 mm. as soon as these areas become distended. A pressure above 40 mm. is very likely to rupture the air sacs. The pressure of 10 mm. established, the rubber tubing to which the canula is attached is clamped off, leaving the lungs distended with the pressure unchanged. A needle attached to a 10 cc. syringe is then inserted obliquely through the tubing between the clamp and the canula and 10 cc. of air is slowly removed from the lungs and is replaced promptly but slowly by 10 cc. of 80 per cent alcohol. This is repeated once or twice so that finally 20 or 30 cc. of air have been removed gradually from the lungs and as much alcohol introduced. The trachea is then tied off and the lungs placed in 80 per cent alcohol for 24 hours, when they are removed, the ligature taken from the trachea and alcohol allowed to trickle from it. No pressure is exerted on the lung, however. The canula is then replaced in the trachea and a pressure of 10 mm. of mercury maintained until the lung is dry. This sometimes requires as long as two days. When the lungs no longer collapse at discontinuation of the pressure, they are considered to be completely dried although they are not necessarily so. They are then ready for the corrosion mass.[25]

a. *Corrosion masses:* The two principal corrosion masses are Wood's metal and celloidin.

1. *Wood's metal:* This substance, an alloy composed principally of tin but containing also bismuth, lead and cadmium, melts at 68 to 70°C. If the lungs have been previously dried, they are buried in warm, dry sand, the molten metal is poured into the trachea through a funnel and the whole cooled as quickly as is feasible. Slight jarring of the sand-box during the injection facilitates the more complete and uniform distribution of the metal throughout the lungs, but there is a danger that the injection may be too complete and massive and thus relationships masked. If fresh lungs are used, and if only the air passages are to be injected, the lungs are removed from the thorax, placed in a dish of moist sand and buried gently in such a way that they are not compressed, with only the trachea unburied. The sand will counteract the tendency of the heavy metal to distend and spread the lung. Into the trachea a small funnel is placed and the molten metal which has been heated to about 72 to 75°C. is slowly poured through the funnel into the trachea. If injection is done with

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[25] Personal communication from Dr. F. L. Reichert.

the lungs in situ, there is no abnormal distention, but the preparation must be cooled quickly in order to avoid the settling out of the metal toward the pendant portions. In the digestion (see below), the preparation should be suspended rather than allowed to lay on the bottom, because the finer divisions are easily broken. This consideration should hold good also for celloidin preparations.

2. *Celloidin:*

Acetone. . . . . .600 cc.
Dried chips of parlodion. . . . . . .48 to 60 gm.[26]

Place the parlodion or celloidin in the acetone and allow it to stand in a stoppered bottle several days until it is completely dissolved, inverting the bottle two or three times a day.

To 200 cc. of the solution add powdered chrome yellow to saturation or until the solution is quite yellow.

To another 200 cc. add 100 cc. of acetone and invert the bottle several times or until the fluid is all taken up in the mixture. Then add Tieman's "soluble blue" or insoluble Prussian blue until a deep blue color is obtained and is uniform throughout the mixture. Dilute the remaining 200 cc. with 100 cc. of acetone and add red mercuric oxide, vermilion or mercuric-sulphide to saturation or until additional pigment produces no further change of color in the mixture.

The first, the thicker, yellow mixture is used for intratracheal injections. The others are for the blood vessels. The lungs are distended through the trachea to 10 mm. of mercury; the vessels are washed out with warm physiological saline containing 4 per cent sodium citrate followed by acetone. Injections are made in one system after the other and are discontinued when the first sign of the mass is seen at the pleura. After the masses in the vessels begin to harden, pressure in the trachea is gradually discontinued, the canula in the trachea is filled with acetone and injection of the yellow mass is made.

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[26] DuPont manufactures a good grade of celloidin under the trade name of *Parlodion.*

The corrosion is sometimes incomplete or is full of blebs. This comes about because of the presence of air and can be obviated by filling each canula with acetone just before injection is to be made (see above); pressure is then turned on and the injection mass allowed to trickle into the canula until it begins to displace the acetone; that is, until all air bubbles are out of the way. The connection is then made tight, and full pressure allowed to work.

b. *Digestion of corrosion preparations:* After the injections are complete the rubber tubes attached to the canulae are tied off, pressure slowly reduced, connection between manometer and bottle broken, all tubes washed out with acetone and the bottles securely sealed or stoppered. The corrosion maintains the normal shape of the lungs if the material hardens while the lungs and heart remain in the thorax. Fifteen minutes suffices for this and at the expiration of this period, the lungs and heart are removed and placed in a large beaker which contains several hundred cubic centimeters of 5 per cent hydrochloric acid. In this the preparation remains for two or three hours at room temperature. Pepsin is then added in amounts of about 1 gm. to every 200 cc., is dissolved by stirring, and the container placed in an incubator at 37° to 40°C. where it remains several days. It is well to remove the corrosion from time to time and wash it by playing a very fine, gentle stream of water from a capillary pipette or needle connected to the spigot. If digestion is not complete in four or five days, the preparation may be placed in water and the remaining tissue allowed to macerate for a few days before the final washing and mounting.

Corrosions may be preserved in glycerine or in a mixture containing two parts each of glycerine and 95 per cent alcohol and one part of water or they may be kept in the dry state. They may be mounted in the medium described on page 272. Wood's metal preparations need no special preservative.

3. *Studying tuberculous tissues in the fresh state:* Certain of the relationships of tubercles to surrounding tissue may

be very satisfactorily studied in the fresh state by means of a binocular microscope and strong direct lighting. Such means also enable one to dissect out small tuberculous areas from the surrounding tissues and study these in a variety of ways, especially by vital staining and tissue culture methods.[27]

a. *Vital staining:* It is very simple to make fresh spreads of tubercles or of tuberculous tissue and study under the microscope the living cells that take part in the reaction. This may be done either with or without the use of a vital dye. Areas are dissected under the binocular microscope and transferred to a small petri dish containing 5 cc. of Locke's solution (see next section) in which they are cut up into small pieces one-fourth to one-half mm. in diameter. If the specimen is to be vitally stained, one drop of a 1 per cent solution of neutral red or the same amount of a 0.5 per cent solution of Janus green is added and stirred into the Locke solution and the mixture allowed to stand for a few minutes. Two or three of the small bits are then taken up in a capillary pipette and deposited on a slide, the excess of fluid is drawn back into the pipette, a cover slip placed, and the preparation is ready for microscopic examination.

While this is not the place to consider in detail what may be seen by this simple procedure, it may be pertinent to remark that the result is surprising and ordinarily quite gratifying. Living epitheloid and other cells show up with extraordinary clearness in such preparations. A description of the findings in the lungs in such a procedure has been given recently by Lewis, Willis and Lewis (130). Dworsky, Smith and Gardner (52) have reported results of the application of the method to peritoneal exudates in tuberculosis.*

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[27] I wish to acknowledge the help in the preparation of this section that has come from the chapter on tissue culture by W. H. and M. R. Lewis in Cowdry's "Cytology" (129) and from suggestions from these authors. There is a good statement of the principles involved, technique employed and results obtained in tissue cultures by Erdmann (56), Strangeways (209), and Fischer (60), each of whom brought out works on the subject of tissue culture technique.

* Very recently Gardner (Proc. Soc. Exp. Biol. & Med., 1927, xxiv, 646) has described a technique of preserving the neutral red of supravitally stained tissues in sections. "(a) Intratracheal injections: 30 to 40 cc. of warm

4. *Tissue culture methods:* Only comparatively recently have tissue culture methods been applied to the study of tuberculosis. In some ways this has been fruitful. Results of such studies have been reported by Smyth (205), Smith, Willis and Lewis (198), Maximow (146), Lewis, Willis and Lewis (130) and others.

a. *Tissue culture fluids:*
  1. *Locke's solution:*

Sodium chloride	0.900 gm.
Calcium "	0.025 "

1:1500 solution of neutral red made up in physiological salt solution is injected into the guinea pig's trachea, which is then tied off. The whole animal is incubated at 37°C. for 5 to 30 minutes. After this interval the dye ceases to react specifically and strains the neuclei. (b) Intravenous injection: 60 to 80 cc. of the same solution are slowly injected into the jugular vein on carotid artery of a guinea pig. If the animal survives it is allowed to live 10 minutes before being killed by air embolism. Occasionally it dies and the whole body is incubated as just described."

Formaldehyde, made alkaline (pH 7.6) with solid NaOH, is kept at 37°C. and 15 cc. is added to 100 cc. Zenker's without acetic acid. In this mixture lungs are kept 12 or 18 hours when thin slices (2 mm.) are cut, washed in a vacuum in fresh Zenkers without acid or formaldehyde, then washed 15 minutes in running water, blotted and put for 10 minutes each into 80% alcohol, 95% alcohol, 95% alcohol and benzene in the following proportions: 9:1, 8:2, 7:3, 5:5, 3:7, 2:8, 1:9. Tissues are then cleared in two or more changes of pure benzene for 1 to 2 hours, are put in benzene saturated with paraffin (in incubator) for one hour and are finally imbedded in paraffin at 56°C. (4 changes in 1/2 hour). Sections are cut and fixed to slide in the usual manner, the paraffin is removed with xylol, and from this point they must be treated individually by dropping the various reagents from dropping bottles. Water is to be avoided. The sections are next treated with absolute alcohol, 95% alcohol with 1% iodine for 15 seconds, several changes of 95% alcohol, and are then carried directly to Harris's haematoxylin (without acetic acid) until stain spreads evenly over the slide, drained and washed quickly in 95% alcohol, followed by water in a beaker, only long enough to thoroughly wet their surface. They are finally dehydrated in 95% and absolute alcohol, cleared in xylol and mounted in balsam.

Intratracheal injection of the dye is irritating and causes migration of cells into the alveoli; so one should become familiar with this cellular response. The intravenous injection of 1% solution of the dye (10 cc. in rabbits and 2 to 4 cc. in guinea pigs, as used by Cash) combined with intratracheal gives very gratifying results.

Potassium “	0.042 “
Sodium bicarbonate	0.020 “
Dist. water	100.00 cc.

2. *Locke-Lewis solution* (127):

Locke's solution	80.00 cc.
Chicken bouillon[28]	20.00 cc.
Dextrose	0.25 to 1.0 gm.

3. *Drew's solution* (51):

Sodium chloride	0.900 gm.
Calcium “	0.020 “
Potassium “	0.042 “
Sodium bicarbonate	0.020 “
Magnesium phosphate	0.010 “
Calcium acid “	0.010 “

To this is added a few drops of embryonic extract, autolized tissue-extract or tumor-extract. One of the extracts is found to be necessary to cultures where continuation by replanting is desired.

4. *Lymph and plasma:* Serum, lymph and plasma have been used as tissue culture fluids. Homologous fluids are better than heterologous ones. Plasma is the most used of these, and it is sometimes diluted (three parts to two of distilled water or physiological saline) since the latter procedure stimulates growth and migration of cells. Blood is collected from the beating heart or peripheral vein of the anaesthetized animal from which tissues are to be taken. It is collected in a paraffined syringe and needle and transferred immediately to paraffined centrifuge tubes which are standing in crushed ice. These are quickly whirled in the centrifuge, and hanging drops are prepared from the plasma before clotting occurs. This procedure is difficult because of the clotting. Heparin has been used to prevent clotting in these circumstances but appears to do the cells some harm.

b. *Culturing of tissues:* The technique involved in preparing tissue cultures is comparatively simple, although aseptic precautions must be strictly employed throughout. For the work, the best quality glassware should be used since in the cheaper glass there is sufficient alkali, even after treatment with acid, to interfere with growth. Cover slips should be meticulously washed and polished in the way these covers are treated for blood smears. They may then be placed in a petri dish and sterilized or they may be placed separately in a horizontal plane in a wire mesh frame and passed several times through a flame immediately preceding use.

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[28] Chicken bouillon is prepared in the same way as beef bouillon with chicken being used in place of the beef.

Tissues intended for cultivation are removed aseptically to a sterile petri dish of about 10 cc. capacity which contains 5 cc. of one of the tissue culture fluids. In the manner already described for preparation of fresh spreads, this is cut into small pieces less than 1 mm. in diameter, and two or three of these are taken up with a little of the fluid into a capillary pipette and transferred to a cover slip. Some of the fluid is drawn back into the pipette so that the particles of tissue adhere in a small drop. When plasma is used as culture medium, the original culture fluid is removed from the cover slip and is replaced by a drop of the former. The cover slip is then inverted over a hollow-ground slide the concavity of which is rimmed with vaseline to prevent drying. The preparation is kept warm and is observed from time to time.

Normal or tuberculous tissues may be grown in tissue culture, and precisely the same technique is employed in either case. Areas of tubercle may be dissected under the binocular, freed largely or completely from normal tissue and cultured. Or normal tissue may be prepared for culture and then infected in either of two ways: Tubercle bacilli, already made into a suspension, may be added either (1) to the culture fluid in which the tissues are cut into pieces; or (2) to cultures at varying periods of time after incubation by substituting the original culture fluid with one containing bacilli. Many of the tubercle bacilli become attached to the tissue or glass. Those remaining unattached may be removed in large part at any desired time by replacing the infected fluid with new fluid containing no bacilli.

Tissue cultures may be kept alive and growing for many years if properly cared for. Subculturing should be done every three days. The old culture should be removed from the cover slip with a small sterile scalpel, washed in Locke's solution, cut into pieces and these planted again on new, sterile cover slips, in plasma to which a little embryonic juice has been added; or the whole culture itself may be replanted. The use of plasma and embryonic extract (made by grinding and pressing chick embryos) is essential to successful subculturing. A discussion of observations upon tissue cultures infected with tubercle bacilli may be found in the reports already referred to (130), (146), (198).

A Few Statements Concerning Allergy and Immunity to Tuberculosis

1. *Evolution of tubercle in normal and allergic animals:* When a normal animal is infected with tubercle bacilli, it reacts in a certain definite way which is characterized by great leisureliness or slowness of response. During the first week there is usually no gross reaction at all, and the animal does not fall ill until several weeks have elapsed. Histologically, evidence of reaction may be seen as early as the fourth or fifth day, and this consists of nuclear activity and proliferation of "epitheloid" cells,—the essential cell in tubercle-formation—at the site of focalization of tubercle bacilli. There are no signs of acute inflammation; *proliferation* is the conspicuous feature of the reaction. This is true where dosage has been small. If animals are inoculated with a suspension containing large clumps of bacilli, the reaction to these clumps is not a pure proliferative one, for, in addition to this, it is featured by a moderate though transient outpouring of polymorphonuclear leucocytes and other attributes of an acute inflammation. But when an animal which has been previously infected and therefore possesses anatomic tubercle is reinfected, that animal responds in a manner markedly different from the normal response just described. At about 10 or 12 hours after the second inoculation, if the dose were large, the animal may become ill so that it sits for several hours in the corner of the cage with its hair on end. With an extremely large dose such

an animal may die within less than 24 hours of the inoculation. At points where bacilli have localized, proliferative reaction is not evident in the early hours of the reaction, but an acute, exudative inflammation is seen in its stead. In these areas there is an outpouring of polymorphs, red blood cells and serum and the reaction rapidly follows the inoculation. After subsidence of this reaction, formation of proliferative tubercle rapidly follows. In the previously infected animal, then, the reaction is rapid and acute (10 to 24 hours) instead of leisurely and delayed (7 to 14 days) as in the animal reacting to first infection; the reaction is not at first proliferative but is almost purely inflammatory; the animal is rendered ill instead of appearing perfectly normal for days after the inoculation. This capacity for "changed reaction" was given the term "allergy" by Pirquet, and the allergic animal is one which has been infected with the tubercle bacillus and possesses the capacity of reacting differently to any subsequent infection with this microörganism or to any of its products.

2. *Relation of allergy to immunity:* The allergic animal not only reacts differently to a second or subsequent infection; it reacts more efficiently, that is, it is relatively immune to the second infection Allergy and immunity appear to go hand in hand, and Krause (111) has referred to immunity as a "function of allergy." This author has compactly summarized the question of the relation between allergy and immunity as follows:

"The 'laws,' if we may so call them, of specific immunity to tuberculosis, as worked out by many experimenters, during the last three decades, may be summarized as follows:

1. There is no specific immunity without preëxisting tubercle.

2. Immunity first appears with the palpable development of the focus.

3. It diminishes with the healing of the focus.

4. It disappears with the enucleation of the focus.

5. The degree of immunity varies directly with the virulence of the immunizing microörganism; or, otherwise expressed, with the amount of tubercle and activity of lesion in the host.

"It is of the utmost significance that the circumstances of allergy exactly parallel those of specific immunity. It is especially significant that, in this respect, allergy stands alone among all known immunological reactions. Precipitins, agglutinins, aggressins, opsonins, complement fixing bodies, anaphylactic hypersensitiveness, etc., are all obtainable without the intervention of tubercle bacilli (and therefore tubercle), and by means of dissociated products. Again, all of these may be lacking in animals, which are demonstrably immune. But as regards allergy, we find that

1. It is never observed unless tubercle is present.
2. It appears coincidentally with the establishment of lesion.
3. It diminishes as lesion heals.
4. It varies directly with the intensity of the disease, which is in turn dependent, not wholly, but in large measure, on the virulence of the bacilli concerned.

"This close and direct parallelism of immunity and allergy at once suggests that immunity is a function of allergy. As to the character of the mechanism, set in motion by the allergic reaction, by which immunity is accomplished, opinions may legitimately differ, inasmuch as the underlying forces of allergy are as yet thoroughly obscure."

3. *Establishment of allergy:* It need not matter in what way an animal becomes infected for the development of allergy because the latter follows the establishment of lesion in any part of the body. There are time-variables depending somewhat upon the situation in the body of the focus or foci of first infection and depending also upon whether the latter was large or small, but the state of allergy or hypersensitiveness develops with developing lesion, regardless of the part of the body involved. The strain R1, described above, is widely utilized as an immunizing agent since it sets up lesion and produces allergy and the immunity that goes with it without impairing the animal's health or causing tuberculosis. A very good routine is to inoculate guinea pigs in the groin with 0.5 cc. of a heavy suspension of R1, from a culture about three

weeks old. It is unnecessary to accurately determine the dose and it is quite satisfactory to use a suspension that is distinctly turbid. After inoculation, the animals are returned to the cages where they should remain for three weeks, during which period one usually palpates the groin once or twice to note evolution of gross tubercle. At the end of this time animals are nearly always allergic. Allergy goes into marked abeyance in advanced tuberculosis, in marked debility, and in several clinical conditions which are but rarely encountered in experimental animals.

a. *Response to dead tubercle bacilli:* It is well known that allergy follows the injection of killed tubercle bacilli. The allergy induced in this way, however, is of comparatively short duration. The histological reaction following injection of dead bacilli was fully described in an excellent paper by Prudden and Hodenpyl (182) in 1891. The reactions from dead and those from living bacilli are indistinguishable in most points, except that of duration and progression.

4. *Determination of allergy: types of reaction:* After an animal develops allergy, it is sensitive not only to tubercle bacilli but to the products of this microörganism. The allergic reaction may, therefore, be elicited by the introduction either of tubercle bacilli or tuberculin into the animal. Reactions are of three types: (1) local, (2) focal, and (3) general. Of these reactions Roemer (187), Baldwin (10), Krause (105) and others have given lucid descriptions and have taken into account the more important theoretical considerations of their nature and evolution. Reference should be had to these papers by one who cares to study this phenomenon.

The *local* reaction is most comonly used as a test of allergy in man or animals. In animals it consists simply of an intracutaneous injection of tuberculin. Dilutions of Old Tuberculin or any other standard tuberculin may be used, but a water extract of pulverized bacilli[29] is preferable because it is free from the ingredients of media and represents essentially a solution of tuberculo-protein. Recently, chemical refinements in the hands of Long (134), Zinsser (242) and especially Seibert (195), have given us an active principle from tuberculin that approaches chemical purity, yet, despite this, its exact chemical nature is still obscure. We get highly satisfactory results by diluting Water Extract with equal parts of physiological saline and injecting 0.2 cc. of the mixture into a shaved area of skin. If toluol has been used as a preservative for the tuberculin, it must be removed, for it is in itself irritating and may produce an inflammation when introduced into the skin. It may be removed by placing the beaker containing the mixture in a pan of hot water and agitating it gently until the odor of toluol has disappeared. Too much heat, however, will cause a fine, granular precipitate to form in the solution and this occurrence must be avoided. The guinea pig will not react to the Pinquet test.

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[29] Since the test for allergy is done with tuberculin, the preparation of a relatively pure tuberculin may be described in this connection. *Water Extract* is the tuberculin commonly used experimentally, and in its manufacture tubercle bacilli are used which have been washed free of media, dried and

If the animal is allergic, there will be a decided area of redness and induration and sometimes central necrosis at the site of injection by 24 hours, certainly by 48 hours, after injection. Necrosis, although not an essential feature of a positive reac

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pulverized (see below). Fifty grams of powdered bacillary material are weighed *under a hood*, placed in a sterile two-liter erlenmeyer flask to which 1000 cc. of sterile distilled water is added. The flask is plugged with cotton and shaken sufficiently for a thorough wetting and suspension of the powder. (Too vigorous or prolonged shaking, however, will produce a true emulsion which is to be avoided.) It is next digested at 48° to 54°C. for 48 hours, being gently shaken three or four times a day, and placed in the ice chest where it is left undisturbed for about two weeks, when the supernatant fluid is carefully decanted and (usually) passed through a Berkefeld filter and layered with toluol. The filtrate is a thin, clear, transparent fluid of rather deep lemon-yellow color. It retains its potency for years.

The strength of this product may be determined by evaporation and weighing of the residue. Two cubic centimeters are placed into each of two carefully weighed watch crystals, which are then transferred to an incubator or oven to dry, after which they, with the dried residue, are again carefully weighed. The calculation is averaged for a cubic centimeter of the extract. This usually amounts to approximately 0.020 gm.

For intracutaneous testing in experimental animals, Water Extract is mixed with an equal part of physiological saline warmed sufficiently for the tion, frequently occurs where the allergy is high, and usually appears the second day after the injection. The redness should measure at least 10 mm. in diameter for the reaction to be called positive. The intensity of the reaction varies with the height of the allergy and, to some extent, also with the amount of pigment in the skin. (Albino skins tend to react with a greater degree of redness and a somewhat less degree of induration than deeply pigmented skins. But these are comparatively slight and should rarely, in themselves, lead to an equivocal interpretation). Allergy remains high for more than a year after R1 infection and may be demonstrated by larger intracutaneous doses of tuberculin for certainly as long as two and one-half years after infection (Willis (235)). Normal animals give no reaction to injections of tuberculin.

The *constitutional* reaction is manifest by illness of the animal. Tuberculin injected intraperitoneally into an infected animal produces, in from 6 to 12 or 15 hours, symptons of illness eventuating frequently in death. The animal loses its body-tone and becomes lax, it shivers and appears

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evaporation of the toluol used as a preservative, and the mixture injected in the amount of 0.2 cc.

The technique of washing, drying and pulverizing tubercle bacilli is also worthy of description in this connection. Liquid cultures are filtered through a Buchner filter in which has been placed the thinnest possible layer of absorbent cotton covered by a piece of cheese cloth. Filtration is done into a side-neck flask under gently suction. Bacilli are then washed with tap water until the filtrate no longer gives a biuret or chloride test. Then suction is maintained over night, or until the mass of bacilli are sufficiently dry to be crumbly when transferred and spread out in thin layers in flat-bottomed porcelain dishes, in which they are dried in vacuo in the presence of sulphuric acid and removed to bottles that are subsequently sealed. The final product resembles "grape nuts" very closely. All these procedures should be carried out under a hood.

These dried bacilli are next ground in a ball mill for at least one week. Care must be taken to prevent any moisture entering the mill for, if this occurs, the bacilli will become tightly packed in a plaque that adheres to the walls of the mill. Thoroughly pulverized bacilli are white in color and have the consistence of flour. This substance may be kept in sealed bottles for a long time, but probably loses much of its active tuberculin-principle after 8 or 10 years. One lot which came under my observation at the age of 10 years was entirely inert. But preparations five years old have been found fully potent.

drowsy, its hair stands on end. It develops diarrhea and is usually dead within 12 or 18 hours of the injection. This reaction is always accompanied by a *focal* reaction that is characterized by a specific inflammation around the foci of tuberculosis in the body. It is supposed, according to the most acceptable theory yet offered in explanation of the reaction (113), that there is a softening of the sclerotic or cellular periphery of the tuberculous area as a result of the focal reaction, and that the rapid absorption of nonspecific, partially disintegrated body-tissues from within the focus is largely responsible for the symptons that go to make up the constitutional reaction (see page 146). For a recently infected guinea pig 300 gms. in weight, 0.20 to 0.25 cc. of undiluted Old Tuberculin will produce a marked reaction that frequently ends in death. If the infection is older than six months, the reaction is likely to be somewhat less severe, but even in an animal infected for as long as a year and a half with R1, this dose will elicit a well-marked, moderately severe reaction but usually will not result in death.

5. *Autopsy-results in reinfected, allergic animals:* Allergic animals fall sick after a dose of tubercle bacilli or tuberculin but, surviving this, they tolerate the infection well. After doses of bacilli that are lethal for normal animals, only slight disease develops (until late). When a group of normal and a group of allergic animals are infected similarly and killed eight or ten weeks later, a very marked and striking difference is observed in the extent of disease. The normals without exception have extensive involvement of all the viscera and of the lymph nodes tributary to the point of inoculation. The allergic group, on the other hand, have enlargement and central caseation of the regional nodes; the spleen is of normal size but may contain one or two areas of tubercle as large as 2 mm. in diameter; the tracheobronchial nodes are occasionally slightly enlarged; the viscera are essentially free of tubercle.

But if these immune animals were allowed to live on, they would, as has been shown by Krause and Willis (117), eventually die of chronic tuberculosis. So the immunity, while real, is only relative and not complete. It delays onset and progress of infection but it does not prevent it in the dosage usually used in experimental work. One cannot escape the feeling, however, that the immunity must be effective and absolute in smaller doses of infection, and this is an important question that should be determined.

CHAPTER XV

HISTOLOGICAL TECHNIQUE

TECHNIQUE OF HANDLING TISSUES

Technique of doing the autopsy has already been given. In saving tissues at autopsy the aim should be to preserve as nearly as may be the integrity and the relationships of all the elements in the given structure, for at best the stained cell in sections is a dead cell, fixed and precipitated, and is one which probably gives but a poor impression of its appearance and structure during life. Tissues having the capacity of elasticity or expansion should not be distorted before or during fixation. It is therefore a good plan in fixing the lungs to do so before opening the chest, where this is feasible. Obviously, examination of these organs in the fresh state is frequently desirable and for that reason the thorax may be opened before fixation. If the lungs may be fixed prior to opening the thorax, a canula is placed through a slit in the trachea and the fixing fluid allowed to run in by gravity until the thorax has reached a moderate degree of distention. Such a manoeuver has this drawback, however, that it is likely to dislodge or distort intra-alveolar exudate. Gentle, rhythmic pressure upon the thorax aids in replacement of air by the fluid. This procedure works beautifully with the rabbit whose lungs distend uniformly and with great ease, but in the guinea pig it is frequently unsatisfactory because the bronchial musculature contracts and renders uniform distention impossible. Many times an entire lobe or even an entire lung cannot be forced "open" even with relatively great pressure. Such irregularities in distention of the guinea pig's lung may sometimes be overcome by distending with air before the introduction of the fixing fluid. The irregularity occurs irrespective of whether fixation is being done with the lungs intact or removed. A simple apparatus for introducing Zenker's fluid into the trachea of these small animals is illustrated in figure 23. Description of the contrivance is given in the legend. Other tissues, such as spleen, liver or lymph nodes, should be cut into pieces not larger than 8 mm. square or, as in the case of the spleen, the entire organ may be incised nearly through at several different places and dropped into the fluid. Lymph nodes or abscesses should be incised before being put in fixing solution.

At times one may desire to save an entire animal for a museum specimen. In such a circumstance the animal may be pinned down to a board after the skin on each side has been laid open. The areas of interest, as the inguinal, iliac or tracheobronchial nodes, are dissected, all viscera placed in the desired position and the whole put in the fixing solution. When such fixation is to be done in Kaiserling's fluid, no time should be lost between exposure of the viscera to the air and fixation of the specimen, because the color of the tissues changes after contact with the air.

1. *Choice of fixing fluids:* Fixing fluids are, of course, numerous, but for tuberculous tissue Zenker's is undoubtedly the best if tubercle bacilli are to be sought in microscopic section. No other fluid allows both bacilli and cells to retain their individuality so well. After formalin-fixation tubercle bacilli stain poorly and are found with difficuly. Bacilli are detected easily in tissues fixed with 95 per cent alcohol, but most people find that it preserves the cells badly although in the hands of Miller it gives satisfactory results.

2. *Fixing fluids: technique of fixation:* Different technical procedures are employed with the different fixing reagents. These will be given for the more important reagents.

a. *Zenker's fluid:* This is an excellent fixing reagent and admits of a great number of cellular and bacterial stains. Bacteria and cellular elements are well preserved. It is prepared in the following manner:

Bichloride of mercury	50 gm.
Bichromate of potassium	25 gm.
Water	1000 cc.

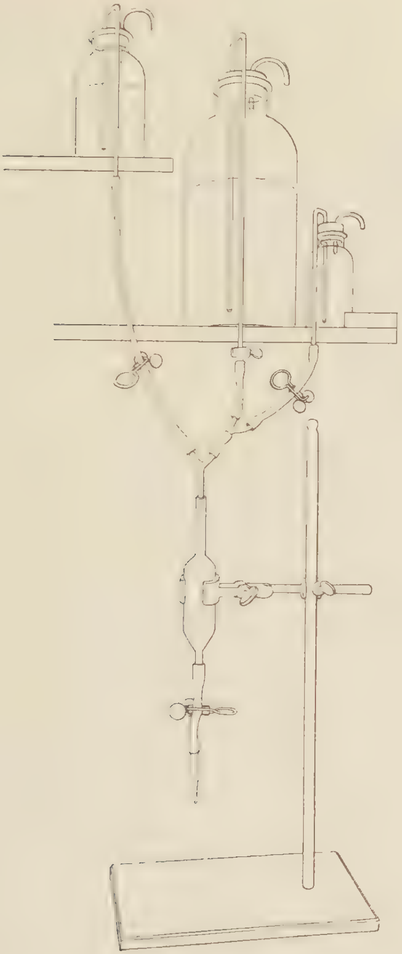

Figure 23. Showing Apparatus for Intratracheal Fixation of Lungs

Zenker's fluid is allowed to flow from the large bottle at the centre into the volumetric pipette. After the pet-cock from this bottle is closed, a little acetic acid is passed into the pipette from the small bottle on the right. The canula connected to the pipette is then introduced into the trachea and the injection made by gravity-pressure. The bottle on the left of the drawing contains water for washing the pieces of rubber hose free of the fluids. This is done by removing the Y-shaped glass tube from the pipette, placing a finger over the end and allowing the water to push first the Zenker's and then the acetic acid back into their respective bottles by opening and then closing first one pet-cock and then the other, while the one on the water-tube remains open

The water is heated and these ingredients slowly added until they are dissolved, stirring being continued meantime. After filtration it is ready for use. A small amount (about 3 cc. to the 100) of glacial acetic acid should be added to the Zenker's *just before it is used*, to facilitate penetration. If the specimen be lungs, which float after the fluid has been introduced, it is well to cover the specimen with cotton and gently push it down. The cotton absorbs fluid and thus forms a wet covering for the specimen. Tissues are placed in the fixing fluid, the jar covered and allowed to set aside for twenty-four hours, when the fluid is poured off and washing with running water begun. This is continued for one, two or three days or even longer, depending upon the size and compactness of the tissue. Lungs are never washed completely free from Zenker's unless each lobe is incised. This is best done after washing has continued for 24 hours or more and is accomplished by cutting with a razor into the depths of each lobe as far as the main stem bronchus, the incision being made at right angles to this structure. When washing is complete, that is, when the water remains clear 24 hours after removal of the tube, 80 per cent alcohol is added and the jar sealed.

b. *Alcohol:* Lungs fixed by intratracheal injection of 95 per cent alcohol should be placed in a jar of alcohol of like strength for two to four hours and then transferred to 80 per cent alcohol for preservation. The trachea is tied off until removal to 80 per cent alcohol. Other tissues should be cut into small pieces not more than 4 to 6 mm. thick and placed first in 80 per cent for three or four hours before being carried over to 95 per cent. These also should be returned to 80 per cent for preservation. Alcohol causes considerable shrinking and marked hardening of tissue, and prolonged stay in higher per cent alcohol produces decided impairment in staining reactions.

c. *Formalin:* Technique of fixation is the same with formalin as with Zenker's. It is generally used in a strength from 4 to 10 per cent, and tissues may be transferred directly to 80 per cent alcohol from it. It does not make tissues as hard and firm as does alcohol. While for certain purposes, as in fixation of tissues for clearing by the Spalteholz method, it is necessary, its use is undesirable, as mentioned above, for fixing tissues in which tubercle bacilli are to be sought, because the latter stain poorly after this type of fixation. Formaldehyde is sold in 40 per cent strength, this being the saturation point of water with the gas.

d. *Kaiserling's fluid:* This is used with great advantage in fixation of museum specimens for preservation of color. Tissues remain from 24 hours to three or four days in solution 1, which contains

Formaldehyde	200 cc.
Water	1000 cc.
Potassium (or sodium) nitrate	15 gms.
Potassium (or sodium) acetate	30 gms.

They are removed at end of fixation and placed in
80 % alcohol for 4 to 24 hours and then in
95 % alcohol for 4 to 24 hours.

(Alcohol causes a return of the colors). Then they are preserved in solution II, which is made according to the formula:

Glycerine	200 cc.
Water	1000 cc.
Potassium (or sodium) acetate	100 gms.

Preparations fixed and preserved by this method should be kept in the dark, as light gradually affects the colors, causing them to turn brown and to lose some of their translucency. Molds sometimes grow on solution II. Addition of a few crystals of thymol or carbolic acid in 0.5 to 1% prevents this, however.

e. *Spalteholz's method of clearing:*

1. Fix in 10 % formaldehyde	24 hours
2. Dehydrate thoroughly:	
a. Alcohol, 80 %	24 hours
b. Alcohol, 95 %	24 hours
c. Alcohol, absolute	24 hours
d. Second solution, absolute alcohol	24 hours

3. Benzene c.p.	12 to 24 hours
4. Oil of wintergreen	Permanently

Very large tissues are easily cleared by this method.

f. *Mounting medium:* In this connection mention should be made of an improved means of preserving museum preparations in a solid medium. The method, proposed by Sudler and Baumgartner (213) is carried out with the solutions and procedure given below.

Solution I:

Potassium acetate	100 gms.
Glycerine	200 cc.
Water	1000 cc.

Solution II:

Gelatin	200 gms.
Solution I	3000 cc.

The gelatin and solution I mixed and brought to a rapid boil with a care not to burn. At 55°C., when the gelatin is about melted, the white of one egg is added for every liter and the solution rendered strongly acid with acetic acid. As soon as boiling is active, the preparation is filtered through two thicknesses of paper into museum jars, which already contain the specimens. A crystal of thymol placed on the surface of the preparation after it has solidified preserves it from molds. The cover should be sealed.

EQUIPMENT

For histological work a certain basic equipment is obviously necessary. This should include, besides reagents, stains, etc., a microscope, a celloidin and paraffin and usually a freezing microtome, paraffin bath, jars for washing and staining, dropping bottles, fiber-blocks, slides, coverslips and instruments for handling sections.

1. *Microscope:* It is assumed that the physician and student has a good microscope equipped with low, high dry and oil immersion lenses and are familiar with the use of the instrument.

2. *Microtomes:*[30] In the earlier days of histological technique, recourse was had to the cutting of free-hand sections, but the introduction of precision instruments has made section cutting much more accurate. There are three types of precision microtome in general use. These are:

a. *Freezing microtome:* Urgent occasion for sectioning tissue removed at operation may arise, and, although a freezing microtome is required but rarely, it should be considered a part of the equipment. It can be set up rather easily by connecting an iron cylinder of liquefied carbon dioxid, such as is used in charging soda fountains, to the freezing box of the microtome. The cylinder is secured firmly in a vertical position, valve down and at about the level of the microtome. Mallory and Wright suggested drilling a small hole in the cap of the escape tube into which a short piece of small bore brass tube is driven. This is connected with the microtome by means of thick rubber tubing which fits securely. In this way a smaller flow of gas is obtained.

These authors also suggest a simple knife made by placing a thick, flat, wooden handle on a blade from a carpenter's plane. For cutting, the blade, which should be kept sharp, is held firmly, bevel side downward, at an angle of 45° to the tracks of the microtome and a little on the bias. Quick but steady and firm pressure is necessary. In freezing, the gas is turned on very slowly and evenly. If specimen is frozen too hard, sections will crumble. A few moments' wait allows a return of the tissue to proper consistence. Sections usually adhere to the knife and can be floated on the surface of water by immersing it at intervals. If thin sections are desired, cutting should be done as rapidly as manipulation of the microtome-screw permits.

b. *Paraffin microtome*: Sections can be cut thinner in paraffin than in celloidin, yet both media are of great value. Several paraffin microtomes are available and there is but little choice between them. The Spencer rotary has met our needs in a very satisfactory manner. The Minot precision microtome

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[30] Modern microtomes of good quality may be obtained from any of the instrument manufacturers, such as Spencer, Bausch & Lomb, Zeiss, Leitz, etc.

(Bausch and Lomb) is designed for either paraffin or celloidin section cutting and yields good results.

c. *Celloidin microtome:* Jung has devised an admirable celloidin microtome in which, by means of screw propulsion, the object is moved along an inclined plane and thus raised to the level of the knife. The other type of celloidin microtome in common use is one in which the object is raised directly by the screw. In each of these the knife is set obliquely at an angle which allows the entire cutting edge to come in contact with the block during its passage. The vertical screw type made by Bausch and Lomb is satisfactory, but for careful serial section making we have had better results from the sliding type.

3. *Paraffin bath:* For blocking tissue in paraffin, the latter must, of course, be in a liquid state. And paraffin may be kept in this state for months if the proper temperature is maintained in an oven or paraffin bath built on the principle of the ordinary incubator under automatic control of a safety burner or bimetallic heat regulator. It should be borne in mind, however, that gradually, over the months, the melting point of paraffin, which is kept liquid, rises.

4. *Centrifuge:* Occasions very frequently arise in the laboratory when a centrifuge is necessary. A water-driven machine is fairly satisfactory and is relatively inexpensive. But an electric "International, size 1 or 1B" is entirely adequate to the needs and is to be recommended.

5. *Washing pans:* When several specimens are to be washed at one time it is convenient to use a large, deep washing pan with rows of hooks soldered into the bottom. Lungs may be tied to the hooks from which they will float as from an anchor. Other tissues, such as liver or spleen, can be enclosed in a cheese-cloth bag and the bag secured to the hook. Such an apparatus is shown and described in figure 24. It has the advantage that the tissues may lie free from other tissues or objects that could compress or otherwise leave a mark, such as is likely to occur especially to lungs if touched.

6. *Jars* or wide-mouthed *bottles* are also useful for washing with running water after Zenker's fixation. A convenient

jar is a one-half pint fruit jar with a glass lid that is held in place by a wire fastener (Mason E-Z Seal). Twenty-four hours after fixation the Zenker's fluid may be poured off and a rubber tube with running water placed in the jar without any disturbance whatever to the tissue. The jar has the advantage of being made air-tight, so that after washing, 80 per cent alcohol may be introduced, the jar sealed and the specimen kept indefinitely in the same container in which it was placed at fixation. Wide-mouthed bottles of from one to 10 ounces capacity are obtainable and are useful for fixing, washing or preserving specimens of tissues of varying sizes. Copper-wire covers for jars and bottles with a hole in the center for the water-tube are valuable in the washing process. Cheese cloth may be used for this purpose, however, by being tied over the top of the container.

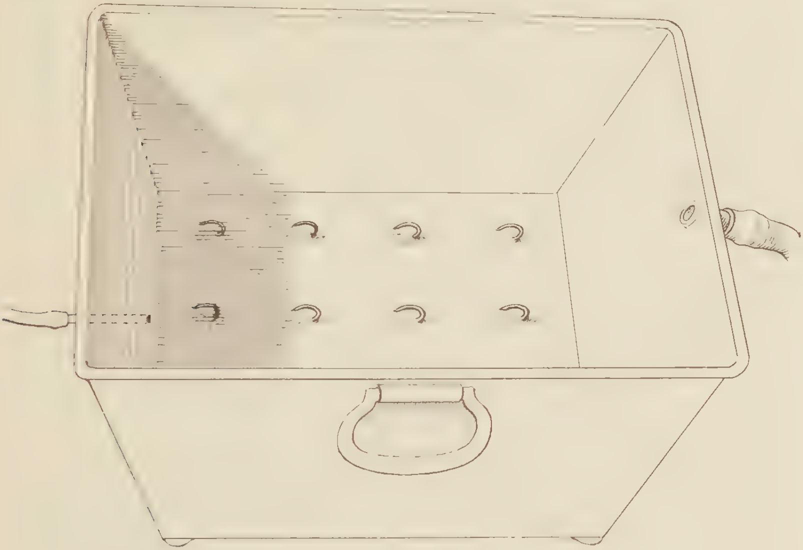

Figure 24. Line Drawing of Washing Pan

The pan is provided with water intake and outflow for a continuous flow of water from the faucet. The hooks on the bottom act as anchors which hold tissues in place during the process of washing.

Staining of paraffin sections is usually done in specially designed *staining jars.* These are of two general types either of which is equally serviceable. One is a tall, rectangular jar with inside measurments a little larger than the usual 1 × 3 inch slide. Two opposite sides are corrugated and can thus hold slides in place. This type is economical of staining reagents, as it is small, and is used preferably where only a few slides are to be stained. The other type is a larger container and sits flat, as a dish, with corrugations at each end for holding the slides which lie on edge. This dish is larger, and has a capacity of twelve 1×3 slides. For larger slides petri dishes are sometimes convenient.

Celloidin sections are stained before they are attached to slides: staining dishes of varying size may be used, depending upon the size of the sheet of sections: small flat-bottomed watch glasses for single sections, petrie dishes for smaller sheets or strips of sections and large crystallizing dishes made after the general style of petri dishes and holding 1000 cc. or more, for staining large sheets of sections.

It is frequently desirable to apply stains or other solutions to single slides or coverslips and this is readily and cleanly done by using dropping bottles of 50 cc. capacity which deliver one drop at a time. Those with a flat and those with a knobbed top are equally useful.

7. *Staining racks:* Staining racks for holding and transferring slides from solution to solution in the staining procedure have been recently devised by MacCallum.[31] They are excellent and can be highly recommended. They are made to hold varying numbers of 1 × 3 or 2 × 3 slides. Figure 25 illustrates one of these racks for 1 × 3 slides. Each groove in the rack has a two-slide capacity, the slides being put back to back.

8. *Slides and coverslips:* The size of slides in most common use is 1 × 3 inches. For serial section work or for mounting large sections, slides 2×3 inches or lantern slides (3 1/4×4 inches) should be used. They should be of clear glass and the surfaces should be perfectly smooth. In tuberculosis work *slides should never be used a second time unless they have been thor-*

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[31] These very useful staining racks are manufactured by the George H. Wahmann Manufacturing Co., Baltimore.

oughly treated with sulphuric acid-potassium bichromate cleaning solution (p. 3) as it is possible for tubercle bacilli to adhere to the slide and be found a second time. The cleaning solution completely destroys bacilli on slides that are exposed to it for 10 minutes. Coverslips should vary in size and shape according to the purpose for which they are required. For work with the high dry objective coverslips of either No. 1 (0.13 to 0.17 mm.) or No. 2 (0.17 to 0.25 mm.) thickness may be used. But for oil immersion work where the focal distance is shorter only No. 1 coverslips can be used. Glassware is best cleaned by washing in tap water without soap, rinsing in alcohol and wiping with a soft cloth, as a freshly laundered, old, worn, linen handerchief.

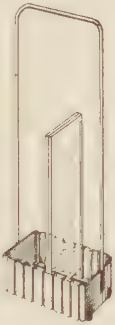

Figure 25. SKETCH OF STAINING RACK

Slides containing sections may be placed back to back in the rack and carried through the various stages of staining without removal.

9. *Blocks:* Tissue which is imbedded in paraffin may be secured to small blocks of hard wood sawed in proper sizes. A convenient way to get a good block is to procure a strip of wood 3/4 × 3/4 inches and 3 or 4 feet long and saw it into rectangular pieces one inch long.

For celloidin mounting, vulcanized fiber blocks ribbed and grooved on one surface are excellent. These may be bought from any house dealing in laboratory supplies.

10. *Instruments:* For handling paraffin sections a small camel's hair brush and a dissecting needle are necessary. For celloidin sections a larger camel's hair brush, a thin, wide, flat,

pliable, metal spatula with a wooden handle and a dissecting needle, are needful.

11. *Running water:* In the laboratory, running water is needed, especially for continuous washing of specimens fixed in Zenker's fluid. An arrangement for this purpose is one in common use, devised by extending an arm from the cold water pipe over a drain board that slopes to the sink's edge. On the arm are numerous stop-cocks to which rubber tubing may be attached, the free end of the tubing being at any desired time available for the jar or bottle containing the specimen to be washed.

Section Cutting

Before the advent of the microtome, tolerably thin slices of hardened tissue were cut free-hand with a razor, but this really fine art has disappeared now that precision instruments are used so universally. For precision cutting, tissues must be infiltrated with and blocked in a firm substance which will preserve relationships: the block must be firmly secured to a rigid base to insure steadiness during cutting and to guarantee sections of even thickness. It is therefore important to block or imbed tissues in substances which answer these requirements, and experience has taught that both paraffin and celloidin meet the needs satisfactorily. Celloidin has a wider range of application, as tissue of large size and almost any consistence may be cut when imbedded in this substance. Paraffin sections, on the other hand, may be cut thinner and thus be more desirable for study of minute cell-structure. Staining of sections in either medium may be done with about the same facility. Actual cutting of celloidin sections requires a longer time than cutting of paraffin sections, but this is offset by the fact that large numbers of celloidin sections can be stained in sheets at one time, whereas with paraffin sections fewer may be stained at a time. Both methods are good.

1. *Selection of tissues:* After tissue has been washed and placed in 80 per cent alcohol, portions of it desired for histological study are selected and cut with a sharp razor. Tissue ranging in size from 1 mm. square up to the entire lungs

of the guinea pig or rabbit, or large pieces of human lung may be imbedded. We have cut serial sections of an entire rabbit's lung imbedded in celloidin without losing a section from faulty imbedding or cutting. After cutting, the desired portion is placed in fresh 80 per cent alcohol for a few minutes and then transferred to 95 per cent, in which it remains from 12 to 24 hours, depending upon its size and density. If the specimen is from the lung, the air must be removed by suction before imbedding takes place. This is done preferably at the 95 per cent alcohol stage. If all air has been removed, the specimen promptly settles to the bottom upon gradual removal of the negative pressure. The specimen is next transferred to absolute alcohol for 12 to 24 hours. Up to this point there is no difference between preparing the specimen for celloidin and for paraffin imbedding. After this point the steps are different and will be considered separately.

2. *Celloidin sections:* a. *Imbedding:* For celloidin-work we have had very satisfactory results with Du Pont's Parlodion. This product comes in thin strips submerged in water. Several days before use it should be dried over night in an incubator and dissolved in a mixture of equal parts of absolute alcohol and ether. Two solutions are used, one of 3 per cent and one of 10 per cent strength. If kept in well-stoppered bottles, these solutions remain good until used. Warthin (224) has lately reported good results from dissolving gun cotton in a solution of equal parts absolute alcohol and ether.

After remaining in absolute alcohol 24 hours, the tissue is prepared and imbedded in celloidin, by treatment with the following solutions:

1. Mixture of ether and absolute alcohol, $\overline{\text{aa}}$, 24 hours.
2. Thin celloidin (3%), from 24 hours to 3 or 4 days, depending upon density of specimen.
3. Thick celloidin (10%), 3 to 10 days.

Meantime, slips of ordinary writing paper or filter paper should have been cut and folded to form boxes of a desired size for blocking the specimen. The boxes are easily made by folding the paper about the bottom, ends and sides of a vulcanized fiber block.

4. Pour a paper box of proper size nearly full of thick celloidin.

5. With forceps place specimen in this and change its position in order to do away with possible air bubbles. Finally, place it so that the surface to be cut faces down, as this will become the top of the block.

6. With long forceps place this preparation in a jar containing a few centimeters of chloroform and allow it to remain several hours or over night. It is better first to place the box-preparation upon a fiber block so that it is in the vapor but not in the chloroform itself. The next morning the block will be rubbery hard. Tear off the paper and trim the block down to proper size with a sharp scalpel. Then mount on the block in the following way:

1. Pour absolute alcohol-ether mixture into two watch glasses. Into one place the celloidin block with the side to be attached to the fiber in the fluid; into the other place a fiber block for dehydration.

2. When the bottom side of the celloidin block begins to get soft and sticky (about 2 minutes), pour thick celloidin over the grooved surface of the fiber block and quickly stick the soft side of the celloidin block to it.

Hold together firmly for a few seconds and add more celloidin if necessary.

3. Harden in chloroform vapor several hours.

4. Remove to 80 per cent alcohol, where mount will keep indefinitely.

In cutting serial sections in celloidin it is necessary to use sugar-plates to hold the sections in their proper relationship to each other until they can be united into one sheet by having 3 per cent celloidin poured over them. For this purpose glass plates 5 × 7 inches must be meticulously washed and freed from any trace of fat and dried with a silk cloth. They are then thinly and uniformly covered on one surface with the sugar solution (see below). In covering the plate one should hold it in a horizontal plane, allowing it to rest on the tips of the thumb and four fingers of one hand, the fingers not touching the edge of the plate. The solution is poured on the plate which is slanted a little to this side and that until the surface is covered, and the plate is next tilted so that the excess solution drains off at one corner and pours or drips back into the bottle or some other container. The solution is made as follows (W. S. Miller's directions):

I. Add powdered candy sugar to 300 cc. boiling distilled water until this solution becomes definitely syrupy.

II. Add dextrine (c.p.) to 100 cc. of boiling distilled water until solution begins to thicken, but remains transparent. Now to 300 cc. of solution I, add 100 cc. of solution II; then add 200 cc. of 95% alcohol: filter.

b. *Cutting*: After eight to twelve hours in 80 per cent alcohol the mount may be placed in the microtome for cutting. Eighty per cent alcohol is allowed to drop from a bottle or funnel on to the block fast enough always to keep it and the knife wet. Sections are cut and transferred from the top of the knife's edge (where they generally remain after cutting) with a camel's hair brush to a petri dish of 80 per cent alcohol from which they may be fixed to slides either by firm blotting or by exposing ether vapor, and the slide then returned to 80 per cent alcohol. But if serial sections are desired, the method which Dr. W. S. Miller uses with conspicuous success should be employed.

Miller removes each section from the microtome to 80 per cent alcohol and here flattens it out, picks it up on a copper spatula and carries it to a piece of filter paper lying in a flat tray and kept wet with alcohol. This paper has previously been ruled into square or rectangular areas, each a little larger than the section. He places each new section in series in the square next to the one just filled. When all the squares are full, he lifts the paper up, turns it over and places it evenly on the sugar plate (above) in such a way that each section adheres to the plate and retains its serial relation to the other sections. He then carefully rolls the paper away from the plate and, with a brush wet with 80 per cent alcohol, touches out all wrinkles. He sets the plate aside for three or four minutes or until it is nearly dry. At this time each section sticks tightly to the plate. He next carefully pours 3 per cent celloidin over the plate of sections, drains this and allows it to dry in the air (about five minutes). He then places this in a large dish of water where the sugar is dissolved, and a sheet of sections floats off from the glass and is ready to be washed and stained. After staining and clearing (p. 285), he cuts the sections from the sheet in series, transfers them in their proper order to slides, 2 × 3 inches, blots them dry and mounts them in balsam. The cutting is admirably done with a sharp scalpel or a paper hanger's cutter. If it is desirable to stain certain of the sections in a different way from others, the sheets may be cut and each portion stained separately or they may be cut and the sections mounted on separate slides before staining is done.

3. *Paraffin sections:* a. *Imbedding:* For imbedding, a good grade of paraffin marked "for imbedding" and having a melting point of 54 to 58°C. is necessary. The prerequisites are serum, slides, and a pan of hot water. The specimen, allowed to stand in absolute alcohol for 24 hours, is treated with the different reagents as follows:

1. Mixture of absolute alcohol and chloroform, $\overline{aa}$, 24 hours.
2. Chloroform (until specimen sinks), 4 to 12 hours.[32]
3. Chloroform saturated with paraffin (in paraffin bath), 24 hours.
4. Paraffin, 2 to 4 or 5 days in the paraffin bath.
5. Another change of paraffin, two days or longer, If there is need for haste, tissue may be blocked after two hours in each change of paraffin.
6. Block in paper boxes, as described under celloidin imbedding. This is best done by pouring the box full of paraffin and transferring the specimen to the box with warmed forceps. Avoid bubbles or remove them. Place downward the surface of the specimen on which cutting is to be done. Work on a warmed copper bar, or better, in the paraffin oven. The block

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[32] Some carry the tissues through alcohol-chloroform mixtures in the following proportions: 75–25, 50-50, 25–75, and finally pure chloroform.

is much firmer and more cohesive if the temperature of the paraffin and tissue is brought to 70°C. before blocking is done. This is desirable if sections must be cut in hot weather. This temperature does no harm to the tissue.

7. Float the box upon cool 90 per cent alcohol and blow gently on the paraffin surface. As soon as this has solidified, immerse the entire block in the alcohol. Cooling in alcohol is a clever dodge of Dr. Miller. It prevents crystallization of the paraffin and makes the block smoother and more homogeneous than is the case when the paraffin is hardened in water.

8. After thorough cooling, trim the block to size and mount it upon a block of wood by heating the surface of the wood and of the paraffin block, pressing the two together for one-half minute and cooling in water. All the opposite borders of the block must be parallel; otherwise, the sections will either not stick together in a ribbon or the ribbon will not be straight.

b. *Cutting:* Cutting of paraffin sections may be very easy or very difficult, depending upon the temperature of the room and the condition of the knife. If the block is hard, the room is too cold. A bunsen flame nearby or an electric heater will soon bring the paraffin to the desired temperature: or if the block is too soft, a short stay in the icebox will answer the need. When the temperature of the room is about 85°F., sections will adhere to one another in long ribbons. These may be run off in lengths of 10 to 18 or more inches and carefully laid out on paper on the table and then cut in shorter lengths and transferred to the surface of a pan of water warmed to a temperature of about 45°C. In doing this, one end of the ribbon is allowed to touch the water first, then quickly but gently the ribbon is stretched and the other end carried down to the water's surface. At this temperature the sections do not melt but become soft and expanded, and lose most of the wrinkles. With a hot needle the ribbon is cut into proper lengths for the slides. Then a slide is thinly covered with serum (p. 284) and wiped off with the hand, placed in water, and moved gently to a position under the ribbon, and lifted up. The ribbon floats on the slide and then attaches. The slides are allowed to stand several hours in the incubator while the excess water evaporates. In cutting, great care should be taken both to maintain an even temperature in the room and to prevent drafts.

In cutting *skin*, as tuberculids, inoculation-lesions, etc., better results follow the use of benzene or oil of cedar instead of chloroform in imbedding with paraffin.

Sometimes entire blocks of tissue are stained before imbedding, but for most work, tissues are very much better stained if done after section than before.

Warthin has suggested a method of cutting sections in paraffin and later converting them into sheets of "celloidin sections" by the intermediate use of molasses plates. For details of this method, which undoubtedly has advantages for certain types of work, the reader should consult the original (224).

The fixative: Egg albumin, so commonly used as a fixative, absorbs and retains certain stains and thus gives an objectionable background to the stained preparation. Serum is a better fixing reagent and may be kept satisfactorily for a considerable time if collected aseptically from the rabbit (or other animal), drawn into small capillary tubes about two inches long, and these sealed at the ends in a flame.

Staining of Tissues

Ordinary staining of tissues is readily accomplished, and the technique will not be given here, since the essential steps are outlined in the next section which deals with the staining of the tubercle bacillus in tissues. If occasion arise for staining tissues, the technique given below is followed except that the carbolfuchsin staining and decolorization is omitted.

Staining the Tubercle Bacillus in Tissues

Tubercle bacilli may exist in scarcity or in abundance in infected tissue. In either case those present may be readily stained, but numerous sections either in series or from different levels of the diseased tissue should be stained because, when bacilli are relatively scarce, none may be found in many of the sections, and reliance should not be placed solely upon one section. The first consideration in such a procedure is, of course, the staining of the tubercle bacillus. But it is also important to stain the tissue in a way that cell-identities can be made out and the histological relationships and environment of the bacillus determined. These desiderata are best realized by staining the bacilli with carbolfuchsin and the tissue with nuclear and cytoplasmic stains.

A very satisfactory method is given below. It can be used equally well for celloidin or paraffin sections, but since the procedures differ, the steps will be given separately for each.

It must be borne in mind that the directions given below are subject to some variation, in the time of exposure, to the different stains, solutions, etc., depending upon the nature of the tissue and the fixative used and the thickness of the sections.

1. *Celloidin sections:* Sections are in sheets in water. Staining solutions should be placed in dishes of suitable size.

1. Alcohol, 80%...........	2 minutes
2. Alcohol, 95% containing 2% iodine crystals (if Zenker's were used)........	15 minutes
3. Alcohol, 95%..........	30 minutes (or until gross iodine has disappeared).
4. Alcohol, 80%..........	1 minute
5. Wash in water	
6. Steam in carbolfuchsin..	1 minute
7. Wash in water	
8. Decolorize in acid alcohol (until red disappears)	1/2 to 1 minute
9. Wash very thoroughly in several changes of water	30 minutes or longer
10. Stain in weak hematoxylin (10 cc. Delafield's in 50 cc. distilled water)....	5 minutes
Or in ordinary hematoxylin......	10 to 20 seconds)
11. Wash again in running water (until blue devel-	

ops)[33] .... 30 minutes or longer
Sheets may remain in tap water over night.

12. Dehydrate and counterstain as follows:
    Alcohol, 80% .... 2 minutes
    Alcohol, 95%, with 2 cc. saturated aqueous orange G to 100cc. alcohol..... 2 minutes
    Alcohol, 95% .... 2 minutes
13. Clear in xylol (after blotting and pouring on fresh xylol several times), or better, in a solution containing:
    (Oil of bergamot.......200 cc.)[34]
    (Oil of cedar...........200 cc.)....2 to 5 minutes
    (Anhydrous carbolic acid 200 gm.)
    (Heat, mix and filter)
14. Cut sheet, place sections on slides in series, blot and mount in xylol-balsam.

2. *Paraffin sections:* Begin with sections dry and adherent to the slide. Then treat in the following way:

1. Heat slowly over gentle flame until paraffin melts. This removes air bubbles and makes for better apposition between section and slide.
2. After cooling, place in xylol.................. 3 to 5 minutes

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[33] Some microscopists prefer staining intensely with hematoxylin and then decolorizing to leave only a deep nuclear stain, before staining with carbolfuchsin.

[34] This solution is one used extensively by Dr. W. S. Miller of Wisconsin. It is very useful in handling celloidin sections since it will clear from 95 per cent alcohol. Celloidin sections cannot be placed in absolute alcohol because of the solvent action of the latter. This clearing solution removes a certain amount of eosin from sections, however, when that dye has been employed.

3. Second jar of xylol	1 to 2 minutes
4. Alcohol, absolute	3 to 5 minutes
5. Alcohol, 95%, containing 2% iodine crystals (if fixed in Zenker's)	15 minutes
6. Alcohol, 95% (two or more changes)	30 minutes (or until iodin is no longer visible grossly)
7. Alcohol, 80%	1 minute
8. Water	1/2 to 1 minute.

This removes the paraffin, takes out the mercury-precipitate of the Zenker's fluid and brings the specimen to water where staining can be done. Then:

1. Steam in carbolfuchsin	1 minute
2. Wash in water	
3. Decolorize in acid alcohol (until red disappears)	1/2 to 1 minute
4. Wash thoroughly in several changes of water	30 minutes
5. Stain in weak hematoxylin (10 cc. Delafield's to 50 cc. water)	5 minutes or longer
(or in ordinary hematoxylin)	10 to 20 seconds
6. Wash in running water until hematoxylin is blue, usually	30 minutes
Sections may remain in water over night.	
7. Dehydrate and counterstain as follows:	
Alcohol, 80%	2 minutes
Alcohol, 95% (with 2 cc. saturated aqueons solution orange G to 100 cc. alcohol)	2 minutes
Alcohol, 95%	2 minutes
Alcohol, absolute	2 minutes

8. Clear in xylol or in Bergamot solution (p. 286)... 2 minutes
9. Blot with filter paper and mount in balsam.

By these staining procedures tubercle bacilli acquire a red stain, nuclei take on a blue-brown and the cytoplasm a faint orange color.*

The objection to the use of acid in the decolorization of tissues has already been mentioned and the scheme of reducing the fuchsin with sodium sulphite as devised by Konrich (101) becomes pertinent especially in this connection. It seems redundant, perhaps, to describe the method again, but for

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* My attention has very recently been directed to the staining method used in the U. S. Army Medical Museum and, through the kindness of Miss Helenor Campbell of that institution, the following formula is given and can be recommended. Tubercle bacilli are deeply stained with red and appear in some sections to be even more numerous than in similar sections stained by the technique given above.

A Differential Stain for Tubercle Bacilli in Tissue

1. Fix tissue in formalin or preferably, in formol-Zenker's solution, imbed in paraffin and cut in thin sections. Remove paraffin from slide by usual method.
2. Stain sections for half an hour at room temperature in Kinyoun's carbolfuchsin (95):

Basic fuchsin........	4 gm.
Phenol crystals.........	8 gm.
Alcohol 95%..........	20 cc.
Water...............	100 cc.

3. Wash in water.
4. Decolorize in acid alcohol (1% hydrochloric acid in 95% alcohol).
5. Wash in water.
6. Stain in alum haematoxylin.
7. Wash in water.
8. Differentiate in acid alcohol (1% hydrochloric acid in 95% alcohol).
9. Wash in water.
10. Mordant in ammonia water 1 1/2%.
11. Wash in water.
12. Counterstain in a saturated aqueous solution of Orange G for 10 minutes.
13. Dehydrate quickly in 95% and then absolute alcohol.
14. Clear in xylol.
15. Mount in balsam.

staining tubercle bacilli *in tissue* the Konrich procedure should have due consideration. It is accomplished as follows:

1. Stain with carbolfuchsin as usual.
2. Wash in 80% alcohol followed by washing in water.
3. Decolorize in a 10% solution of pure crystals of sulphite of sodium, freshly made, 1 to 2 minutes.
4. Wash in water.
5. Stain for 5 minutes in hematoxylin (10 cc. Delafield's in 50 cc. water).
6. Wash again in water, and
7. Place in 70% alcohol.

From this point, dehydration, counterstaining, clearing and mounting are done in the same manner as in the methods given above.

The method is useful in handling paraffin sections because the acid used in decolorization in the Ziehl-Neelsen technique acts upon the serum-fixative with the result that sections sometimes tend to become dislodged from the slide. It is likewise useful in handling sections in either paraffin or celloidin if they are to be stained with hematoxylin, because it is difficult to be certain that all the acid after decolorization has been removed from the tissue. If a trace of acid remains in the tissues, it will slowly, in the course of months, act on the hematoxylin to turn it red and thus completely mask the red bacilli and make their identification difficult or impossible. This misfortune is by no means inevitable in the use of acid decolorization but relatively prolonged washing must be done if it is to be averted. The use of sodium sulphite instead of acid obviates the necessity of prolonged washing and does away with an uncertain factor in the staining procedure.

Staining Other Bacteria in Tissues

It becomes necessary at times to stain other bacteria than tubercle bacilli in cut sections, and fortunately there is a quick and comparatively easy method devised by MacCallum (141) by which both gram-positive and gram-negative bacteria may be stained in the same section. The procedure is as follows:

Tissues should be fixed in Zenker-formaldehyde solution and very thin paraffin sections used.

"1. Stain for from ten minutes to one-half hour or more in Goodpasture's stain, which is:

	Gm. or cc.
30 per cent alcohol	100.00
Basic fuchsin	0.59
Anilin	1.0
Phenol crystals	1.0

2. Wash in water.

3. Differentiate in 40 per cent formaldehyde solution. This requires only a few seconds. The bright red color washes away and gives place to a clear rose.

4. Wash.

5. Counterstain in saturated aqueous picric acid. The section remains until it assumes a purplish yellow, about three to five minutes or less.

6. Wash in water.

7. Differentiate in 95 per cent alcohol. The red reappears and some of it is washed out. Some of the yellow of the picric acid is also washed out.

8. Wash in water.

9. Stain in Stirling's gentian violet five minutes or more. (p. 295).

10. Wash in water.

11. Gram's iodin solution.

12. Blot dry without washing.

13. Anilin and xylene (equal parts) until no more color comes away.

14. Two changes of xylene.

15. Balsam.

Gram-negative organisms stain red, gram-positive blue. The tissues stain brilliantly in shades of red and purple. Fibrin is, of course, sharply stained, and the granules of mast cells are prominent as red or purplish coccus-like bodies. . . ."

STAINING OF CONNECTIVE TISSUE, RETICULUM AND ELASTIC TISSUE

One will not infrequently desire to stain connective tissue, reticulum and elastic tissue. The following procedures are very good:

1. *Connective tissue.* For this Van Gieson's method is excellent if tissues are fixed in Zenker's fluid. Standard reagents required are

    1. One per cent aqueous solution acid fuchsin.
    2. Saturated aqueous solution picric acid. When needed, add 5 cc. of the fuchsin to 100 cc. of the picric acid.

1. Stain deeply with alum-hematoxylin.
2. Wash in water and stain for 5 minutes in the acid fuchsin-picric acid mixture described above.
3. Dehydrate in 95% alcohol and (quickly) in absolute.
4. Clear in xylol
5. Mount in balsam.

2. *Reticulum.* Foot (J. Lab. & Clin. Med., 1923–1924, ix, 777) has modified the Bielchowsky method in such a way as to give excellent results (Zenker's fixation).

After paraffin has been removed with xylol, the Zenker fluid with iodine, the latter taken out with 5% hypo (thiosulphate of sodium) and the sections washed in water, the following steps are carried out:

1. Potassium permanganate, 0.25% . . . . . . 5 minutes.
2. Wash in water
3. 5% oxalic acid . . . . 15 minutes.
4. Wash in distilled water
5. 2% silver nitrate (in subdued light) . . . . . 48 hours.
6. Wash thoroughly in distilled water.
7. Ammonium-silver bath (prepared as follows) . . . . 30 minutes.
    20 cc. of 10% $AgNO_3$+20 drops 40% NaOH (made from aq. dest.).
    Wash precipitate out and decant.

Dissolve with strong ammonia water drop by drop, leaving trace of precipitate.
Filter.
Bring to 80 cc. by addition of distilled water.

8. Wash in distilled water.
9. 5% formalin . . . . 30 minutes.
10. Wash in tap water.
11 1% aq. gold chloride . . . . 1 hour.
12. Wash in tap water.
13. 5% hypo . . . . 2 minutes.
14. Wash for several hours.

At this stage, sections may be dehydrated and mounted, or the following steps may be taken, for better contrast:

1. Weigert's iron-hematoxylin . . . . 1 minute.
2. Wash until blue.
3. Van Gieson's stain . . . . ½ minute.
4. Avoid water and dehydrate quickly with 95% and absolute alcohol.
5. Clear in xylol and mount in balsam.

Reticulum is black or deep violet, connective tissue rose or red and nuclei brown to black The method is much less formidable than it appears.

3. *Elastic tissue.* Unna's method is very satisfactory. The stain contains the following:

Orcein	1.00 gm.
Hydrochloric acid	1.00 cc.
Absolute alcohol	100.00 cc.

1. Stain tissues over night in this solution, or over a low flame for 15 minutes (until solution thickens).
2. Wash well in 70% alcohol and then in water.
3. Dehydrate in 80%, 95% and absolute alcohol.
4. Clear in xylol.
5. Mount in balsam.

Elastic fibers are deep brown and connective tissue fibers pale brown.

Stains and Reagents Most Commonly Used in Tuberculosis Work

1. *Carbolfuchsin*: Neelsen's original formula remains the best.

Fuchsin (for bacilli)	1.0 gm
Dissolved in 5% aq. carbolic acid	100.0 cc.
Absolute alcohol	10.0 cc.

2. *Acid alcohol:*

Alcohol, 95%	97.5 cc.
Concentrated HCl	2.5 cc.

3. *Loeffler's methylene blue:*

Saturated alcoholic (95%) methylene blue	30 cc.
Potassium hydrate solution, 1–10,000	100 cc.

The saturated alcholic solution should be kept as stock. Either solution remains potent a long time.

4. *Hematoxylin:* Delafield's hematoxylin is entirely satisfactory.

Dissolve 4 grams hematoxylin in 25 cc. of 95% alcohol and add this to 400 cc. of saturated aqueous solution of ammonia alum (aluminum and ammonium sulphate).

Let stand in loosely covered beaker or unstoppered bottle for about a week in the light. Then filter and add 100 cc. glycerine and 100 cc. 95% alcohol and continue "ripening" process in the light until the color assumes a purplish tinge. Refilter and keep indefinitely (until color becomes red, when it is no longer good). Refiltration is necessary occasionally.

5. *Orange G:* Make a saturated solution in water and keep as stock.

When needed, add 2 cc. of this to 100 cc. 95% alcohol.

6. *Iodine:*

Iodine crystals	2 grams
Alcohol, 95%	100 cc.

7. *Methyl violet:* Usually used in 1% aqueous solution; is a good nuclear stain.

It is frequently desirable to make histological study of tuberculous tissue irrespective of the presence of bacilli. This may be done by staining with hematoxylin and eosin or even better perhaps by use of the stain next described.

8. *Unna's alkaline methylene blue:*

Methylene blue	1 gm.
Potassium carbonate	1 cc.
Water	100 cc.

When used with eosin this is a beautiful stain. The procedure as given by Mallory and Wright is as follows:

1. Aqueous eosin, 5%.... 20 minutes or longer
2. Wash in water
3. Unna's alkaline methylene blue, diluted 1 to 4 or 5 in water.................. 10 to 15 minutes
4. Wash in water
5. Differentiate and dehydrate in 95% alcohol, keeping in constant motion meantime. When pink color returns and nuclei are still blue (under microscope)
6. Place quickly in absolute alcohol
7. Xylol
8. Xylol balsam.

The 95 per cent alcohol for differentiation must contain colophonium which is present in alcohol kept in the barrel but not in that kept in glass. These authors suggest keeping in stock a 10 per cent solution of colophonium in absolute alcohol, a few drops of which are added to the 95 per cent alcohol at the time of differentiating. If celloidin sections are used, fresh 95 per cent alcohol is added after differentiation is complete and sections are immediately blotted and xylol added. Blotting and addition of xylol should be repeated until sections are clear.

9. *Stirling's gentian violet:*

Gentian-violet	5.00 gms.
Alcohol, 95%	10.00 cc.
Aniline	2.00 cc.
Water	88.00 cc.

10. *Other stains sometimes used:* Other stains frequently used in general tissue work and after carbolfuchsin are:

a. *Picric acid:* Used in strength from 1/2% to saturated aqueous solution

b. *Malachite green:* Made in saturated solution and used in the following strength:

Saturated solution	50 cc.
Water	100 cc.

c. *Bismarck brown:* Commonly used in 1% aqueous solution. Stronger dilution can be made by boiling and adding the dye to saturation (3%).

d. *Eosin:* Eosin can be used as a counterstain where bacilli are stained if used in great dilution. Some use methylene blue and weak eosin after carbolfuchsin and get beautiful pastel shades which do not interfere with the finding of tubercle bacilli: it is often needed with hematoxylin for tissue stain and with Unna's methylene blue.

Eosin comes in two forms: (1) water soluble and (2) alcohol soluble. It is a very powerful stain and keeps a long time in 1% to 5% strength aqueous solution and indefinitely in 1% strength in 95% alcohol.

e. *Safranin:* May be made (with heat) either as saturated aqueous solution (Safranin O) or as a mixture of equal parts of saturated aqueous and saturated alcoholic soluble safranin O. Both water soluble and alcoholic soluble forms are purchasable.

11. *Method for blackening table tops:* Mallory and Wright have given an excellent method for blackening table tops. It follows:

Solution I

125 grams copper sulphate
125 grams potassium chlorate } Boil until dissolved
1000 cc. water

Solution II

150 grams anilin hydrochlorate
100 cc. water

Use white wood for tops, since it is cheaper and takes stain better than pine. Oak gives good results.

Spread paper to cover floor completely under and around tables.

1. Apply with brush two coats of Solution I *while hot;* the second as soon as the first is dry.
2. When second is dry, apply in the same way two coats of Solution II and let them dry thoroughly.
3. Put on with a *cloth* thin coat of raw linseed oil and polish thoroughly by rubbing.
4. When dry wash thoroughly with *hot soapsuds.*
5. Rub again with raw oil.
6. Rub with cotton waste over and over again until the black does not come off. This requires hard work.

To keep tops in perfect condition, rub off once a week with white (liquid) petrolatum.

N.B.—If table tops are blotched with paraffin wax, or resin, remove these before beginning to paint. Stains do not harm. Paint top surface and edges only.

CHAPTER XVI

EQUIPMENT FOR A TUBERCULOSIS LABORATORY

In compiling the subjoined list of equipment and materials an attempt has been made to indicate the appointments of a well ordered laboratory of tuberculosis-research. The list may appear too long in certain sections. If this be true, it is because more articles are listed than merely those with which one might barely get along. On the other hand it is obvious that, in a disease of such varied facets as tuberculosis, special studies would require special equipment not cited here. But in general the effort has been made to indicate those items that might be needed in an ordinary laboratory of research character. With each article is given an approximate value which is based on the price of similar articles in current catalogues of houses dealing in such materials. The list has been compiled from the inventory sheets of the Laboratory of the Kenneth Dows Tuberculosis Research Fund.

Article	Type	Size	Value
Animal tags	Aluminum	½ inch diameter	$ 3.00 per 100
" cages	Strong wire with removable tin bottoms		12.00 to 15.00 each
" cage stands	To accommodate 6 cages		20.00 each
Am. Standard Incubator	Board of Health	25 x 30 x 18″	300.00 "
" " "	paraffin bath	24 x 16 x 14″	200.00 "
" " dry sterilizer		18 x 24 x 14″	60.00 "
Arnold steam sterilizer	rectangular	14 x 9 x 9″	75.00 "
Asbestos mats		8 x 8″	3.50 doz.
Autoclave	vertical	24 x 11″	85.00 each
	horizontal	16 x 24″	350.00 "
Balance	analytical		60.00 "
"	trip		15.00 "
" weights	analytical	1 mgm. to 100 gms.	15.00 "
" "	trip	1 gm. to 1 kgm.	10.00 "
Ball mill	bacteria grinder	1 liter	15.00 to 30.00
" " pebbles	porcelain	½ to 1″ diameter	0.60 lb.
Beakers	pyrex	30 to 2000 cc.	0.15 to 1.00 each

Bell jar	glass	9 x 15″	5.00 each
Blower	foot-power	7-8″ diameter	12.00 “
Boilers	double	2 or 3 quart	3.00 “
Boilers (or pots)	aluminum	10 quart	3.50 “
“ wash	copper		10.00 “
“ fish	enameled	16 to 24″ long	5.00 “
Bottles	amber	1000 cc.	0.05 “
“	aspirator	3 gallons	4.00 “
“	balsam	30 cc. ±	0.25 “
“	dropping	50 cc.	0.40 “
“ culture	Blake—narrow mouth	8 oz.	1.10 doz.
“ narrow mouth	mushroom stopper	2 to 16 oz.	0.40 to 0.80 each
Bottles, specimen	wide mouth	30 to 300 cc.	0.08 to 0.15 each
“ “	extra wide mouth	3 oz.	0.15 each
“ sputum	without neck	1 to 2 oz.	5.00 gross
Boxes	pasteboard	1 x 2 x 3½″	0.25 doz.
“ wooden	for slides	1 x 3″ and 2 x 3″	0.20 each
Brushes	bristle		0.50 “
“	test tube	varying	1.00 doz
Burettes	for pinchcock	50 cc.-1/10 calibration	1.25 each
“	with pinchcocks	“ “	2.00 “
“ support	Chaddock		3.00 “
Burners	Bunsen		1.00 “
“	micro		1.00 “
“ blast	Bunsen		5.00 “
“ radial	Fletcher		3.00 “
“ solid flame	“		2.00 “
Cassaroles, porcelain	Coors	85 to 95 mm. diameter	0.75 “
Centrifuge, complete with metal guard	water-motor		15.00 “
“ electric, complete	International	No. 2	375.00 “
“ tubes	plain and graduated—pyrex	15 cc.	3.00 doz. and (grad.) 1.00 each
“ “	plain—pyrex	50, 100 cc.	5.00 to 6.00 doz
Clamps	Bunsen	9″	0.50 each
“	Hoffman	1/2 and 3/4″	0.30 each
“	Mohr's pinchcock	2″ to 3″	0.15 “
Counting chamber	double Neubauer		10.00 “
Condenser	Liebig	400 mm. long	4.00 “
Corks	XXX	No. 0 to No. 24	0.38 to 4.50 doz.
Cork borer	brass	1/8″ to 9/16″	3.50 each
“ press	rotary		1.50 “
Crucibles	porcelain, s.c.p.	No. 0 to No. 3	0.15 to 0.45 each
Culture dishes	Petri	50 x 10; 100 x 15; 150 x 15	0.25 to 0.60 pair
“ “	moist chambers	200 x 240 x 70	1.75 each
Culture flasks	(see Blake bottles)		
Culture tubes	test tube—no lip	150 x 16 mm.	2.50 per 100
Cylinders	double graduations	5 cc. to 1000 cc.	0.40 to 2.35 each
Dessicators	Scheibler	5″ to 10″ diameter	1.50 to 8.00 “
“	Freiling	10″ diameter	20.00 each
“ plates for	porcelain	2 to 8 holes	1.00 to 2.00 “
Dishes, porcelain	Coors No. 00 to 8	70 to 215 mm. diameter	0.25 to 1.50 “
“ crystallizing	glass	200 mm. diameter	1.25 each
“ preparation	“	36 mm. diameter	0.25 “

Dissecting instruments			
Forceps	bone	8″ long	2.50 "
"	long-pronged	10 to 18″ long	0.75 to 1.50 each
"	dissecting	3 to 6″ long	0.25 to 0.50 "
"	mousetooth-lock	4 or 5″ long	1.50 each
"	ordinary lock	4 or 5″ long	1.50 "
Scalpels	several types		1.00 "
Scissors		3 to 10″ long	1.00 to 3.50 each
Section lifter	copper	20 mm. and 35 mm. blade	0.50 each
Probe	Mall		0.35 each
Needles			0.40 doz.
Cartilage knife		6 or 7″ long	1.50 each
Aneurism needle			1.00 "
Hemostats		4 to 8″ long	1.00 "
Artery clamps		1½ to 2″ long	0.75 "
Electric motor	A.C. or D.C.	1/6 H. P.	40.00 "
Embedding boxes	lead	10, 20 and 30 mm. high	0.75 "
Fermentation tubes	Am. P. H. Standard		0.50 "
Filters	Berkefeld and Mandler	From 1 3/8 x 5/8″ to 10 x 2″	1.15 to 4.00 each
" mantels for		From 2 1/2 x 1 1/8″ to 14 x 4″	0.50 to 1.75 "
Filter paper		75 to 330 mm. diameter	0.15 to 1.25 per 100
" "	folded	320 mm. diameter	2.75 " "
Flasks, pyrex	Florence	50 cc. to 2000 cc.	0.15 to 1.75 each
"	Erlenmeyer	"	0.15 to 2.00 each
" filtering	side-neck	1000 cc.	1.00 each
"	volumetric	500 cc.	1.50 "
Funnels, glass		1 to 6″ diameter	0.20 to 0.75 each
" "	ribbed	6 to 10″ "	0.50 to 2.00 "
" "	Buchner	3 to 6″ "	1.75 to 6.50 "
" "	separating	400 cc.	4.00 each
" stands		12″ long	1.00 "
Glass tubing	thick walled	6, 8, 10 mm. diameter	0.50 lb
Ice box			
Incubator (see Am. Standard Incubator)			
Jars	Standard and Am. Med. Museum	3 to 12″ high	0.60 each
Measuring apparatus	caliper	4″ long	1.00 "
Mechanical stage			30.00 "
Microscopes	compound		150.00 "
"	binocular		180.00 "
"	dissecting, binocular		120.00 "
Microtomes	rotary		200.00 "
"	Minot		300.00 "
"	sliding: Jung		200.00 "
Micro-cover glasses	No. 1	18 mm. sq., 35 x 62 mm. 22 mm. sq., 43 x 70 mm.	1.50 to 2.20 per oz.
Microslides	non-corrosive	3 x 1 and 3 x 2″	2.20 per gross
"	hollowground	3 x 1″	1.20 per doz.
Microslide boxes	see boxes		
Mortars and pestles	glass	2 to 4 oz.	0.50 each
" "	porcelain	150 to 200 mm. diameter	3.00 "

Optical goods			
camera lucida			25.00 "
double demonstration eyepiece			40.00 "
projection lantern			70.00 "
Paraffin bath	see Am. Standard Incubator		
Pipettes	serological	0.1 cc. to 25 cc.	0.60 "
"	volumetric	5 to 200 cc.	0.15 to 0.75 each
Pipette boxes	copper	2 x 2 x 20″ 1 3/4 x 2 1/2 x 16″	3.00 to 4.00 each
Platinum-Iridium wire	fused	1/4 to 1/2 mm. diameter	0.50 inch
" loop holder	aluminum	3/16 x 8″	0.25 each
Razor			3.50 "
Reading glass		4″ diameter	4.00 "
Rings	cast iron	2 to 5″ diameter	0.20 "
Ringstands	" "	12 to 20″ high	0.10 "
Rubber stoppers	solid and perforated, with 1 and 2 holes	No. 3 to 9	1.50 pound
" tubing	laboratory	5/16″ bore x 1/8 wall 1/4 ″ bore x 1/8 wall	0.15 ft.
" "	pure gum	4 mm. bore 1 3/4 wall	0.10 ft.
Spatulas	steel	5 or 6″ blade	1.00 each
"	porcelain with spoon at one end	8″ long	0.75 "
Staining dishes		capacity 10-3 x 1 slides	0.45 "
" "	Coplin	capacity 4-3 x 1 slides	0.40 "
" racks	MacCallum	6 compartments	2.50 "
Supports (see under clamps)			
Syringes	Record	1 cc. to 20 cc.	2.50 to 6.50 each
"	Randall-Faichney	1 cc. (graduated in	1.50 each
	Sub Q No. 2021	1/100ths.)	1.50 "
"	Tuberculin, B-D	1cc. " "	1.50 "
Syringe needles, steel	Record & Luer	Guage 27, 25, 22. 1/2, 5/8, and 1″ long	1.50 doz.
" " "	" "	1 to 2″ long 21 to 17 guage	2.00 "
Test glasses	conical	100 and 200 cc.	0.40 each
Test tubes (see under culture tubes)			
Test tube baskets	wire	4 x 5 x 6″	1.00 "
" " supports	copper—Wassermann racks	6 x 3 1/2 x 2″	1.50 "
Thermometers		100 to 250°C	1.00 to 1.50 each
Titration outfit	LaMotte		40.00 each
Transfer holders (see under platinum wire)			
Trays (pans)	aluminum	11 x 14 x 1″	1.00 "
"	enamel	10 x 20 x 2″	1.00 "
Triangles	pipe stem	2″	0.60 doz.
Tripods		4 1/2 to 9″ diameter and 9″ high	0.50 each
Tripod rings			0.50 set
Tumblers		8 oz.	1.75 doz.
Warming table	copper bar		2.50 each
Watch glasses	"crystals"	1 1/2 to 5″ diameter	0.75 doz.
" "	Syracuse	2 5/8″ diameter	1.50 doz..
Water bath	copper	8″ diameter	15.00 each
" "	electric Wassermann	13 x 14″	60.00 "
" " racks		6 x 2 x 2″ high	3.50 "

Weighing bottles		40 to 50 mm. high	0.25 “
Wire gauze	copper	4, 5, and 6″ square	0.10 to 0.20 each
“ “	iron	4, 5, and 6″ square	0.50 doz.

Reagents

Acetone			0.40 lb.
Acid, acetic			0.85 “
“ glacial acetic	c.p.		0.67 “
“ carbolic	“		0.25 “
“ hydrochloric	“		0.40 “
“ nitric	“		0.45 “
“ picric, crystals	“		1.25 “
“ pyrogallic			0.25 oz.
“ sulphuric	comm.		0.05 lb.
“ “	c.p.		0.40 “
Agar Agar			1.50 “
Alcohol, absolute	ethyl		4.50 gallon
“ 95%	“		3.00 “
Alum carmine solution			0.40 oz.
Aluminum ammonium sulphate	U.S.P.		0.20 lb.
Ammonium hydrate	c. p.		0.45 “
Anilin (anilin oil)	“		0.90 “
Anilin blue	alcohol soluble		0.10 gm.
“ “	water “		0.10 “
Balsam, Canada	paper filtered		3.25 lb.
Benzol	c.p.		0.40 pint
Bismarck brown	Grübler		1.00 for 20 gms.
Brom thymol blue	La Motte	0.04 alcoholic solution	6.00 for 100 cc.
Calcium chloride	c. p.		1.00 lb.
Carbolic acid (see acid, carbolic)			
Carbolfuchsin			0.40 bl. oz.
Carmine			0.40 “ “
Celloidin (parlodion)	Du Pont		1.00 oz.
Chloroform	c. p.		1.10 lb.
Chromic acid			1.00 “
Chrome yellow			0.10 “
Copper acetate			1.25 “
“ sulphate			1.25 “
Dextrine			0.40 “
Dextrose	Bacto		3.50 lb.
Eosin	water and alcohol soluble		5.50 for 100 gms.
Ether	U.S.P.		0.65 lb.
Formaldehyde	40%—U.S.P.		0.36 “
Fuchsin	acid		5.60 for 100 gms.
“	basic		5.60 for 100 gms.
Gentian violet			0.40 oz.
Glycerine	c.p.		0.40 lb.
Hematoxylin			5.00 for 25 gms.
Heparin	Hynson, Westcott & Dunning		7.00 gm.
Indulin			0.50 for 10 gms.
Iodin			0.50 oz.
Litmus paper	red and blue		0.15 vial
Malachite green			0.75 for 10 gms.
Martius yellow			0.50 for 10 gms.

Mercuric bichloride		1.20 lb.
“ oxide	red	1.30 “
“ sulphide	red	2.60 “
Methyl violet		0.75 for 10 gms.
Methylene blue		0.75 for 10 gms.
Nigrosine		0.50 for 10 gms.
Oils, anilin (see anilin)		
Bergomot		8.00 lb.
cedar	for immersion	4.50 “
cedarwood		1.50 “
origanum		6.25 “
wintergreen, synthetic		0.75 “
Orange G.	water soluble	0.60 for 10 gms.
Oxalic acid, crystals	c.p.	1.25 lb.
Paraffin, for embedding	M.P. 50 to 56°C.	0.15 lb.
Pepsin		0.35 oz.
Peptone	Diffco	5.00 lb.
Phosphomolybdic acid	c.p. crystals	1.00 oz.
Picric acid (see acid)		
Picrocarmine		0.50 gm.
Potassium bichromate	c.p.	0.45 lb.
“ carbonate	c.p.	0.70 “
“ chloride		0.70 “
“ hydrate	c.p.	0.60 “
“ nitrate	c.p	0.30 “
“ sulphate	c.p.	0.50 “
Rochelle salts	c.p.	
Saffranin		0.75 for 10 gms.
Sodium acetate	c.p.	0.40 lb
“ bisulphate	c.p.	0.50 “
“ carbonate	c p.	0.50 “
“ citrate	c.p	1.15 “
“ nitrate	c.p.	0.50 “
“ sulphate	c.p.	0.40 “
“ sulphite	c.p.	0.45 “
Starch		0.15 “
Thymol, crystals	c.p.	0.50 oz.
Tieman's soluble blue		3.50 for 100 gms.
Tin chloride		1.20 lb.
“ oxide		2.00 “
Toluol		0.40 oz.
Turpentine	rectified	0.50 “
Wright's stain		0.50 “
Xylol		0.40 lb

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